

The use of RAFT polymerisation in the synthesis of polymeric micelles for targeted drug delivery to macrophages

A thesis submitted by:

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Declaration of Originality

I, Katherine Montgomery, hereby confirm that I conducted the research presented in this thesis under the supervision of Doctor Matthew J. Fuchter at the Department of Chemistry, Imperial College London and Associate Professor Susan K Nilsson and Doctor John Chiefari at the Commonwealth Scientific and Industrial Research Organisation, Melbourne, Australia. The work described herein is entirely my own unless otherwise stated.

London, 23rd March 2016



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Abstract

The aim of this project was to develop a series of polymeric micelle particles for application as selective drug delivery agents, specifically to tumour associated macrophages. In Chapter II RAFT polymerisation was applied to the synthesis of amphiphilic block copolymers and the self-assembly of polymers into micelle particles was subsequently studied under aqueous conditions. Since previous studies have shown that the size of the particle is crucial for selective phagocytosis by macrophages, a series of micelles of different sizes were prepared by altering the polymer composition and chain length. The size of the particles synthesised ranged from 15 nm to nearly 200 nm. In Chapter III conditions were developed to fluorescently label the micelles with rhodamine B to allow for tracking *in vitro*. Crosslinking conditions were also developed to increase micelle's stability *in vitro*. A succinimide monomer was incorporated into the hydrophilic section of the polymer, once assembled the micelles were shell-crosslinked using a diamine linker. In Chapter IV cellular studies comparing macrophage and non-macrophage uptake were performed to determine promising candidates for selective drug delivery to macrophages. Micelles were successfully endocytosed by macrophages, however, it should be noted that micelles were also endocytosed by non-macrophage cells. Micelle size did not have an effect on endocytosis within the range tested but some micelles were endocytosed more than others. A fluorescent dye was encapsulated inside micelles so that the micelle and cargo could be independently tracked. The fluorescent dye was successfully delivered to cells. The reproducibility of results was analysed and the most promising candidate for drug delivery was identified.

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For my wonderful parents

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Abbreviations

$\bar{D}_M$	Molecular weight dispersity
4-NH	4-needle head
ACVA	4-cyanovaleric acid
AF647	Alexa Fluor 647
AIBN	2,2'-azobisisobutyronitrile
AM	Acrylamide
AN	Acrylonitrile
ATRP	Atom transfer radical polymerisation
BHT	2,6-di-tert-butyl-4-methylphenol
BM	Bone marrow
BMA	Butyl methacrylate
BODIPY	Boron-dipyrromethene
br	Broad
BSA	Bovine serum albumin
BV510	Brilliant violet 510
CD45	Cluster of differentiation 45
CDCl₃	Deuterated chloroform
CMC	Critical micelle concentration
CMT	Critical micelle temperature
CSF-1	Colony stimulating factor- 1
CSF-1R	Colony stimulating factor- 1 receptor
CSIRO	The commonwealth scientific research organisation
d	Doublet
DAPI	4',6-diamidino-2-phenylindole
dd	Doublet of doublets

DLS	Dynamic light scattering
DMA	Dimethylacrylamide
DMAc	<i>N,N</i> -dimethylacetamide
DMF	Dimethylformamide
dn/dc	Refractive index increment
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EPR	Enhanced permeability and retention
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FSC-A	Forward scatter- area
FSC-H	Forward scatter- height
GPC	Gel permeation chromatography
HPLC	High performance liquid chromatography
LCST	Lowest critical solution temperature
m	Multiplet
MA	Methyl acrylate
MAA	Methacrylic acid
Mac-1	Macrophage-1 antigen
MALS	Multi-angle light scattering
MCF	Mean channel fluorescence
M_n	Number averaged molecular weight
M_p	Peak molecular weight
MRhB	Methacryloxyethyl thiocarbamoyl rhodamine B
M_w	Weight averaged molecular weight

MW	Molecular weight
MWCO	Molecular weight cut-off
NaHCO₃	Sodium bicarbonate
NaN₃	Sodium azide
NaNO₃	Sodium nitrate
NAS	<i>N</i> -acryloyloxysuccinimide
NHS	<i>N</i> -hydroxysuccinimide
NIPAM	<i>N</i> -isopropylacrylamide
NMP	Nitroxide-mediated polymerisation
NMR	Nuclear magnetic resonance
PBS	Phosphate buffer solution
PEG	Poly(ethylene glycol)
PEGMA	Poly(ethylene glycol) methacrylate
PERI	Peritoneal
PI	Propidium iodide
PMMA	Poly(methyl methacrylate)
ppm	Parts per million
PSt	Polystyrene
q	Quartet
RAFT	Reversible addition-fragmentation chain-transfer
RCF	Relative centrifugal force
RFU	Relative fluorescence units
rh	Rhodamine
s	Singlet
siRNA	Small interfering ribonucleic acid
St	Styrene

t	Triplet
TAM	Tumour associated macrophage
THF	Tetrahydrofuran
UV-Vis	Ultraviolet-visible spectroscopy
VAc	Vinyl acetate
w/w	Weight per weight

Chapter I

Introduction

1.1 Drug delivery

Drug delivery is currently an area of huge importance in the treatment of numerous diseases; many drugs have been proven to be efficient *in vitro* but are not delivered sufficiently or selectively to the desired location *in vivo*.¹⁻⁴ A common problem, particularly with regards to cancer treatments, is that a drug is delivered to the required tissue but is also delivered to either the surrounding tissue or to similar cell types. In chemotherapy, for example, drugs are often targeted to cancer cells by taking advantage of their fast proliferation, however, other cell types with fast proliferation are also targeted; i.e. cells which make up the skin, hair and the lining of the mouth.⁵ When large percentages of a drug are delivered off target high doses are required to ensure enough of the drug is delivered to the target cells. To reduce unwanted side effects and the need for high doses targeted drug delivery systems with higher specificity are needed for the treatment of cancer and many other diseases.⁶

1.1.1 Nano drug delivery systems

Nano drug delivery systems are currently an area of great interest. The aim of nano drug delivery systems is to improve a drug's performance. The end goal is the safe, fast and effective delivery of drugs to a specific tissue in the body. As with all medicinal treatments, cost is also an issue that has to be considered for the therapy to have long-term feasibility. Many issues associated with delivery can be combatted using a nanocarrier drug delivery system. For example, nanocarriers can improve selectivity, regulate dosage over extended periods of time, reduce the amount of drug that needs to be administered, reduce drug toxicity, extend the half-life of administered drugs and overcome solubility issues.^{7,8} Many drug delivery systems currently in development have not only achieved selective delivery, but also more efficacious delivery to the target cells.⁶ Nanocarriers have proven their utility in the diagnosis and treatment of many diseases, particularly in the treatment and prevention of cancer.^{5,9-16} For example, Kataoka *et al.* conjugated the anti-cancer drug doxorubicin (trade name Adriamycin) to a poly(ethylene glycol)-poly(aspartic acid) block copolymer to give a micelle-forming polymer-drug conjugate. Their results indicated that this doxorubicin-polymer micelle had, not only less toxic side effects than doxorubicin, but also higher anti-cancer activity.¹²

Although there are many advantages to using nano-carrier drug delivery systems, there are a number of issues these carriers need to overcome to achieve successful drug delivery (**Figure 1**). For instance, it is common for particles, or conjugated drug systems, to be excreted from the body *via* the kidneys.^{15,17} Nano-carriers have an advantage over other, smaller drug delivery systems as > 20 nm is generally regarded as large enough to avoid excretion *via* the renal pathway.^{5,6,18} Another advantage micelles have over other nano delivery systems is that micelles formed from amphiphilic block copolymers have a hydrophilic outer shell. This also helps deter the particles from being excreted *via* the renal system.⁵ Kataoka *et al.* found that drug-polymer conjugates, which assembled into micelles, remained in the blood at much higher concentrations than the drug, doxorubicin, alone.⁵ The polymer-drug micelles also had lower organ accumulation than the drug. Lower organ accumulation indicates less non-specific uptake. As well as the renal system, the reticuloendothelial system is also known to remove particles from the blood before they can reach their target site. In the reticuloendothelial system phagocytic cells work to engulf, breakdown and clear damaged or old blood cells as well as particles and bacteria from the blood.¹⁹ The reticuloendothelial system is primarily made up of macrophages (which are discussed in detail in section **1.2**) and monocytes.²⁰

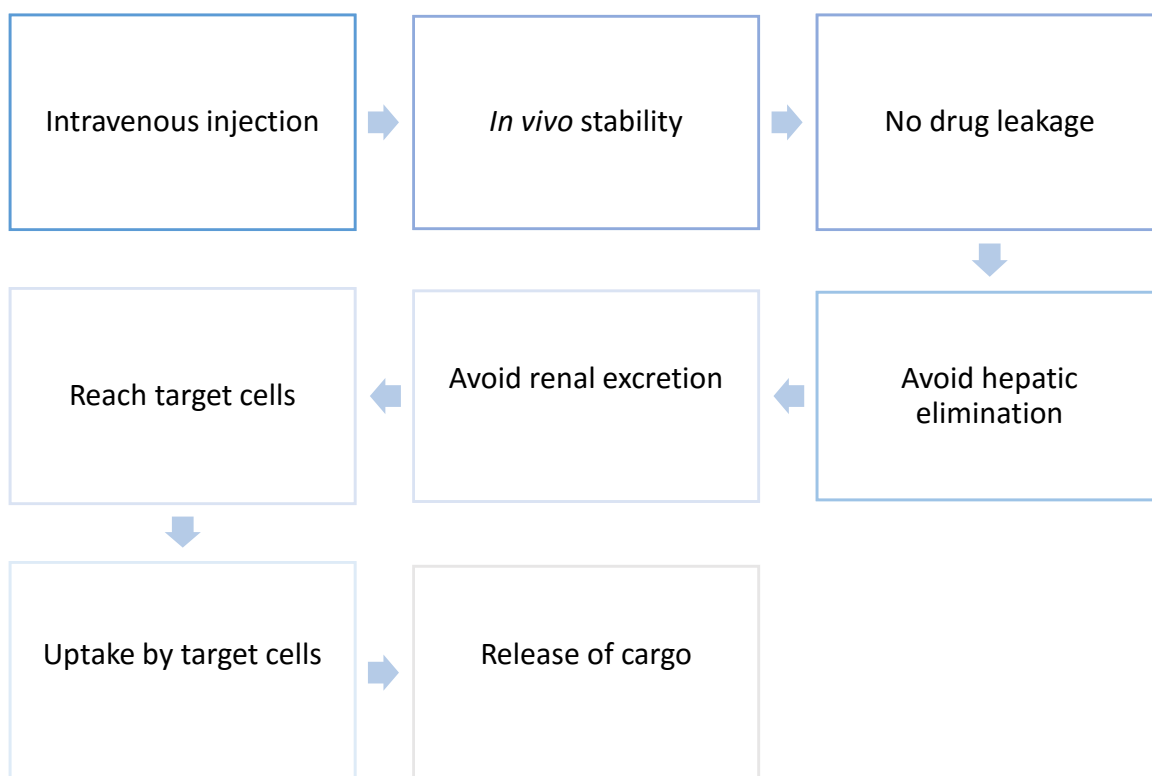
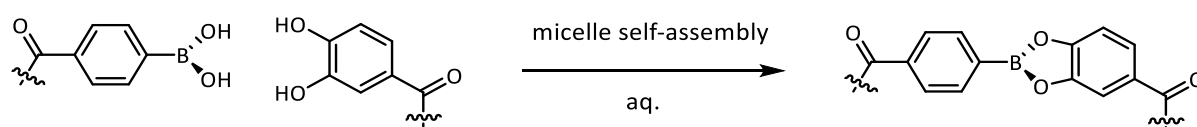


Figure 1. Challenges which must be overcome for a drug delivery system to be successful.

Polymeric micelle particles have been an area of interest in the nano drug delivery field over the last few decades. Success stories involving polymeric micelles as delivery vehicles include the encapsulation and delivery of hydrophobic drugs, proteins and DNA fragments.^{8,11,16,21–34} One of the main factors which gives micelle delivery systems an advantage over other, similar structures is the adaptability of micelles and the level of control afforded over every aspect of the particle. Tuning the structure of the core of the micelle allows it to be adapted specifically for different cargo. This means that the encapsulation and rate of release can be optimised for a wide range of drugs and other cargo such as genes or dyes. Chemical modifications to the shell of a micelle particle enables more specific delivery, allowing certain organs or cell types to be targeted. This is a huge advantage as, in principle, more targeted drug delivery means a higher efficacy and less side effects.

An issue with using micelle particles for drug delivery is that they can disassemble at low concentrations. If the polymer concentration is too low then the polymer will exist as unimers as opposed to micelles, this is discussed further in section 2.1.1. As it is likely that micelles will

be used at very low concentrations (nM- μ M) *in vitro* and *in vivo*, micelles that disassemble under these conditions are not suitable for drug delivery purposes. If micelles disassemble before they reach their target destination then the drug will not be delivered to the target site and instead will be released elsewhere in the body, potentially causing harmful side-effects. This issue of instability has been overcome by the crosslinking of micelle particles, which ensures their stability *in vitro* and *in vivo*. For example, Lam *et al.* crosslinked micelles assembled from block copolymers of poly(ethylene glycol) and dendritic cholic acids by incorporating boronic acid and catechol (**Scheme 1**).³⁵ The boronic acid and catechol were incorporated in separate polymers and reacted together when the micelles were assembled under aqueous conditions, crosslinking the micelles *via* a boronate ester linker. Crosslinked micelles were found to be stable at much lower concentrations than uncrosslinked micelles. When the drug paclitaxel was loaded into the micelles it was released quickly (over 20% in 5 h) from the uncrosslinked micelles but much slower from the crosslinked micelles (approximately 5% in 5 h). Lam's crosslinking strategy stabilised the micelles for use in *in vitro* and *in vivo* and helped minimise premature drug release.³⁵ Other delivery systems, such as liposomes, can also be structurally unstable and fragile when circulating in the blood for extended periods of time, making crosslinked micelles a more practical option.⁵



Scheme 1. Lam *et al.* incorporated 4-carboxyphenylboronic acid and 3,4-dihydroxybenzoic acid into separate polymer chains prior to micelle assembly. The groups were crosslinked during micelle self-assembly to give boronate ester crosslinked micelles.³⁵

A number of micellular delivery systems have reached clinical trials.^{13,36} These include Kayaku *et al.*'s doxorubicin micelle carrier (NK-911), assembled from a poly(ethylene glycol)-*b*-poly(aspartic acid)-*b*-doxorubicin block copolymer (**Figure 2**), which has been found to be efficiently delivered to solid tumours in mice, and Ranson *et al.*'s micelle-doxorubicin system, made from pluronic (triblock copolymers consisting of a hydrophobic polyoxypropylene block with poly(ethylene oxide) hydrophilic blocks on either side) block copolymers to treat refractory cancers.^{25,26,37}

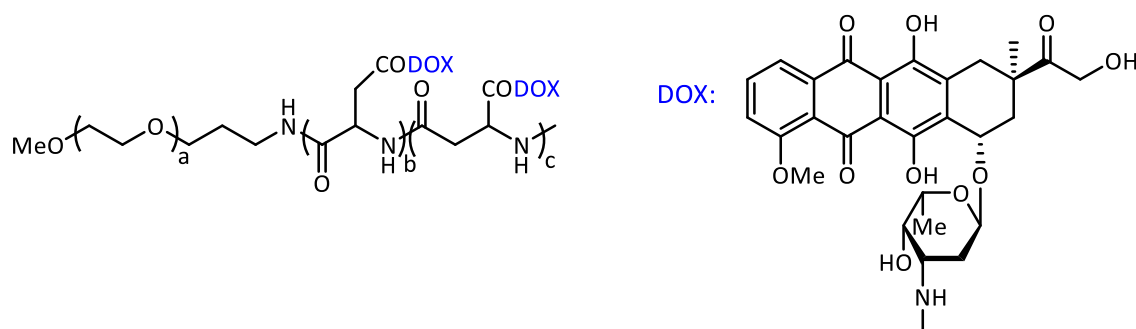


Figure 2. Structure of block copolymer poly(ethylene glycol)-*b*-poly(aspartic acid)-*b*-doxorubicin used to make NK-911. Free doxorubicin was also encapsulated in the micelle.²⁵

1.2 Macrophages

Macrophages are a type of phagocyte; white blood cells that protect the body by ingesting pathogens, cell debris and other molecular targets.^{38,39} The term phagocyte translates from the Greek as ‘cell eater’ and it encompasses any cell that has the ability to phagocytose (engulf) foreign matter or dead cells. Phagocytes are essential in the body as they fight off infections and ensure that dead cell debris does not damage healthy tissue. Other phagocytes include monocytes, neutrophils, dendritic cells and mast cells.

Macrophages differentiate from monocytes and are found in a large number of tissues in the body. Macrophages found in different tissues vary significantly and are subsequently known by different names. Kupffer cells are macrophages found in the liver (alongside stellate macrophages) and their role involves reacting to various stresses placed on the liver such as infections and toxins.⁴⁰ Alveolar macrophages meanwhile, are found in the lungs and their role is to engulf and destroy any harmful particles that have infiltrated the lungs.⁴¹ Other macrophage locations include connective tissue (histiocytes), bone marrow, neural tissue in the brain (microglia), the spleen, lymph nodes, the gut and serous cavities.⁴² As well as varying between different tissue types, macrophages also vary greatly within each tissue as they can exist in several states of activation. The size and role of a macrophage can change depending on its state of activation, macrophages can be classified as M1 or M2. M1 macrophages primarily cause tissue damage and prevent cell proliferation while M2 macrophages promote tissue repair and cell proliferation. M1 macrophages work to fight anything harmful to the host while M2 macrophages work to fix anything damaged in the host.⁴³ The distinction between M1 and M2 macrophages which causes the resulting difference in activity is that M1 macrophages predominantly produce nitric oxide which is

involved in suppressing cell proliferation and M2 macrophages predominantly produce ornithine which promotes cell repair and proliferation (**Figure 3**).^{43,44}

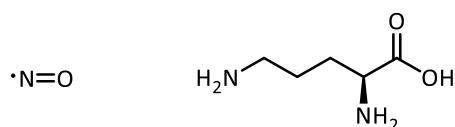


Figure 3. Nitric oxide (left) predominantly produced by M1 macrophages and ornithine (right) predominantly produced by M2 macrophages.

As mentioned above, macrophages differentiate from monocytes. Monocytes exist in the blood and in the bone marrow, they differentiate from myeloblasts in the bone marrow. Myeloblasts differentiate from myeloid progenitors which differentiate from hematopoietic stem cells (**Figure 4**).²⁰ Monocytes are formed in the bone marrow and then enter the blood stream within 24 hours. Once in the blood stream, monocytes can travel to different organs where they differentiate into tissue-specific macrophages or they can remain as monocytes for one to two days.²⁰ If after this time they have not been recruited to a specific tissue the monocyte will die and be removed from the body.⁴⁴ Signals from the microenvironment and tissue surrounding a monocyte determines what type of macrophage it will differentiate into and its activation state.⁴⁴ Recruitment and differentiation are regulated by growth factors. Growth factors known to be involved in this process are colony-stimulating factor-1 (CSF-1) and interleukin-34 (IL-34).⁴⁴⁻⁴⁶

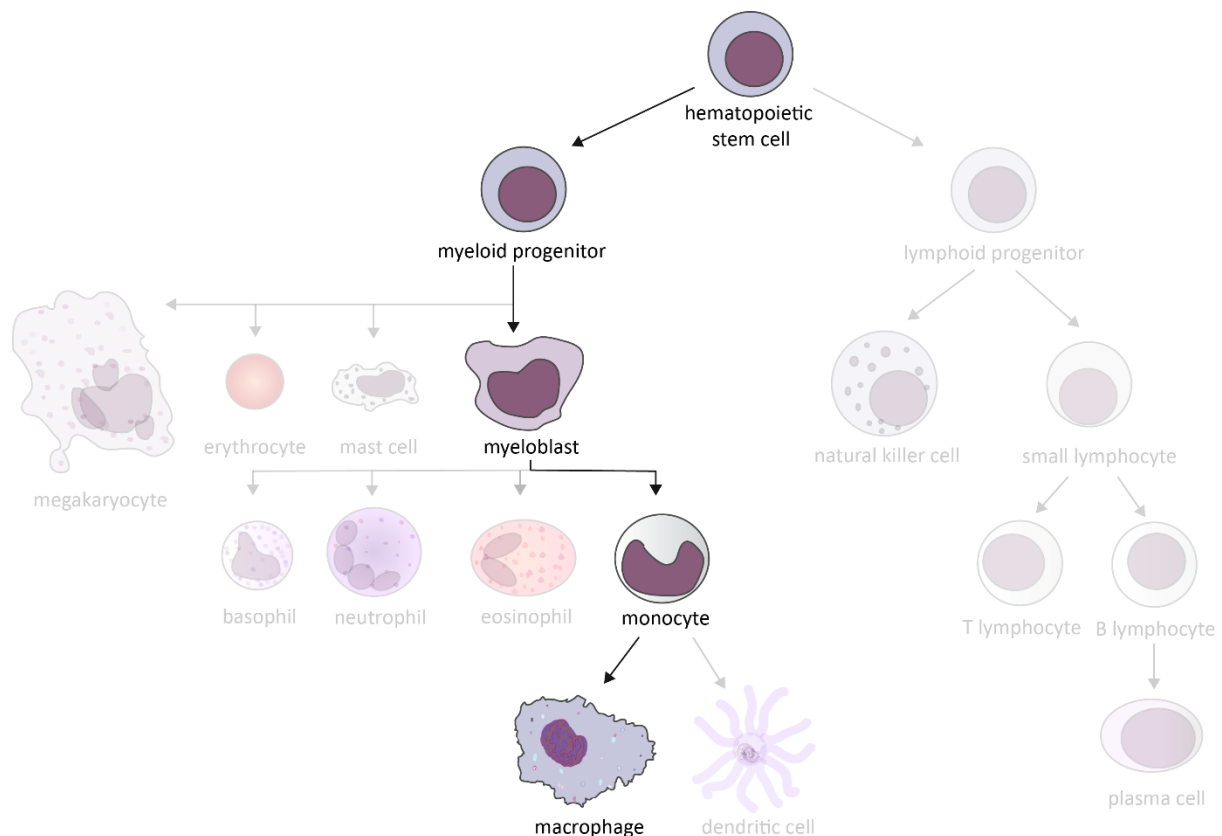


Figure 4. Differentiation from hematopoietic stem cell to macrophage in the bone marrow.

The primary role of a macrophage is to phagocytose foreign matter in the body.⁴⁷ The process that enables macrophages to recognise which particles, or debris, they need to phagocytose is known as opsonisation. Opsonin molecules adsorb to the relevant particles, labelling them for phagocytosis. These opsonins help the particle attach to the macrophage and trigger phagocytosis. Opsonin is a Greek word, which means ‘to make more tasty’. It is macrophage surface receptors that bind to opsonins in the first step of phagocytosis.⁴⁸ Macrophage surface receptors include the mannose receptor, which recognises terminal mannose and fucose sugars (**Figure 5**) found on proteins. Scavenger receptors are also found on macrophages, these recognise apoptotic cells as well as carbohydrates and lipids in bacterial walls. Macrophages also have Fc receptors which bind to immunoglobulin G-antigen labelled cells (such as bacteria and viruses) or debris and help trigger phagocytosis as well as cytokine production. Adhesion receptors, such as CD18/11b are also found on macrophages. Adhesion receptors are responsible for helping macrophages connect to other cells.⁴⁸

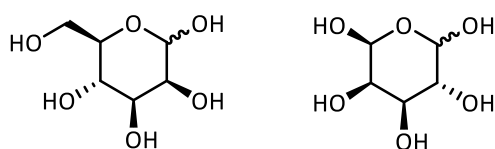


Figure 5. D-mannose and D-fucose sugars, recognised by the mannose receptor.

Macrophages also play a large role in the adaptation of the immune system to help the body recognise and overcome various infections. Macrophages do this *via* antigen presentation which involves macrophages displaying a portion of the matter they have engulfed on their surface to other cells in the immune system. Macrophages engulf the foreign matter and break it down into peptides which are transported to the cell's surface by major histocompatibility complex glycoproteins. Once at the cell's surface the peptides are displayed to lymphocytes.⁴⁹

Although macrophages are largely defined by their ability to phagocytose it is not their only method of uptake, they are also capable of other types of endocytosis such as receptor mediated endocytosis, caveolae dependent endocytosis and pinocytosis, these are discussed further in section 4.1.1.^{50–52} Macrophage's different uptake mechanisms are an important consideration when designing targeted delivery systems.

1.2.1 Why target macrophages?

Macrophages have been found to be involved in a wide range of diseases from arthritis to acquired immune deficiency syndrome (AIDS). The relationship between macrophages and cancer is one in particular that has been studied at length. A brief summary of macrophage's roles in different diseases is outlined in this section, with a focus on macrophages in cancer.

Macrophages have been found to be involved in rheumatoid arthritis, a type of autoimmune disorder.^{53,54} In rheumatoid arthritis joints inflame and can be very painful, making simple movements difficult. The macrophages involved in this disorder are chronically activated synovial macrophages which can cause inflammation as well as joint destruction, worsening both the symptoms and the long-term damage of the disease.^{53,54} It is believed that reducing the number of pro-inflammatory macrophages in patients would be an effective treatment for rheumatoid arthritis.⁵⁴

In AIDS macrophages can internalise the HIV virus, *via* binding to the CD4 receptor and second membrane receptor CCR5. Macrophages then transport the virus around the body, acting as a distributor of the infection.^{55–57} Targeting these macrophages would potentially help destroy the HIV virus.

Macrophages also play a role in inflammatory bowel diseases such as Crohn's disease as they regulate inflammation in the gut by engulfing pathogens and dead cells.^{2,58} In patients that suffer from inflammatory bowel diseases the macrophages have assumed a different phenotype. Instead of reducing inflammation by phagocytosing pathogens, the macrophages release pro-inflammatory cytokines such as interleukins 1, 6, 8 and 12 and tumour necrosis factor- α .⁵⁹ The ability to target and influence intestinal macrophages would result in great benefits for treating patients with inflammatory bowel diseases. Current therapies for inflammatory bowel diseases, such as steroids, are untargeted and can be toxic to other cells in the body.²

Leishmaniasis is another disease which macrophages play a part in. Studies investigating the transmittance and survival of the *Leishmania donovani* bacteria have revealed that this parasite lives and prospers inside macrophages found in the spleen and liver.^{60,61} Targeting these macrophages could have huge treatment benefits when combating this disease.

Similarly to AIDS and Leishmaniasis, the airborne bacteria which causes tuberculosis (*Mycobacterium tuberculosis*) is also internalised inside macrophages, specifically alveolar macrophages in the lungs. The bacteria can survive in macrophages unthreatened and multiply within the macrophage.^{62–64} Therefore, targeting macrophages is an important part of treating tuberculosis. Other infectious agents are also phagocytosed by macrophages and can survive once they have been engulfed including *Legionella pneumophila* and *Salmonella typhimurium*.⁶⁵

A rare disorder, known as Gaucher disease, requires macrophage targeting for treatment to be successful. In Gaucher disease macrophages with high numbers of lipids are found in the lungs and in bone as well as in the liver and spleen. For treatment of the disease to work, β -glucocerebrosidase needs to be delivered to macrophages.⁶⁶

Macrophages in cancer

Hanahan and Weinberg defined six characteristics needed for malignant tumour growth to occur, the six hallmarks of cancer. The characteristics are: self-sufficiency from external growth signals, insensitivity to negative growth signals, resistance to apoptosis, limitless replication potential, sustained angiogenesis and acquisition of tissue invasiveness (**Figure 6**).⁶⁷ Hanahan and Weinberg also emphasise the importance of the role of the micro-environment in tumour development. Malignant tumours require a specific micro-environment and macrophages have been found to be predominant in this micro-environment.^{68,69}

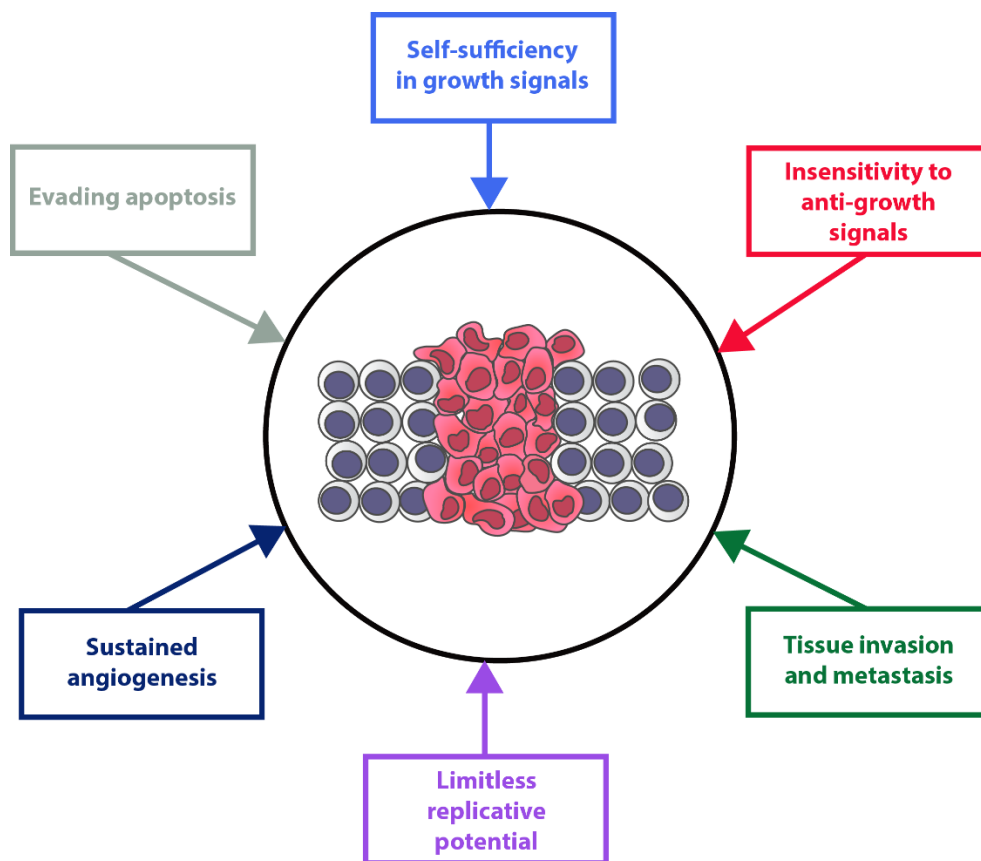


Figure 6. The six hallmarks of cancer.⁶⁷

Tumour associated macrophages (TAMs) are one of many potential cellular targets to which drugs could be selectively delivered using nano drug delivery. Results from clinical and experimental studies strongly suggest that TAMs are involved in the development of cancer by promoting malignant tumour growth and metastasis, they contribute to angiogenesis, matrix breakdown and tumour-cell motility.^{70,71} A clinical study carried out by Leek and Harris

has shown that there is a correlation between the presence of macrophages at a tumour site and the occurrence of angiogenesis.⁷² Macrophages are present at the location of basement-membrane breakdown at the beginning of tumour development, this is where the tumour progresses from being benign to malignant and from where the growth of more advanced tumours initiates. Tumours essentially 'hijack' macrophages and use the macrophage's matrix remodelling abilities to invade the surrounding tissue.^{70,73,74} A study carried out by Condeelis and Segall, using multiphoton imaging, found that interactions occur between tumour cells, macrophages and blood vessels. This further supports the idea that tumours are able to manipulate macrophages which are recruited to the tumour site in response to the release of CSF-1 from the tumour cells.⁷⁵ Additionally, macrophages have been found to release mutagenic compounds during inflammatory response processes which play a role in cancer initiation and promotion.⁷⁰

CSF-1

Macrophages require colony stimulating factors to be able to develop, proliferate, differentiate and survive.⁷⁶ The macrophage colony stimulating factor, CSF-1, has been found to be over expressed in ovarian, uterine, breast and prostate tumours and over expression correlates with poor prognosis.⁷⁰ It is believed that TAMs are present in the tumour micro-environment as a result of their enlistment by chemokines and CSF-1. CSF-1 binds to the receptor, CSF-1R, resulting in the phosphorylation of tyrosine residues residing in the intracellular kinase domain of CSF-1R. This tyrosine phosphorylation triggers docking and phosphorylation of downstream signalling proteins which are responsible for the differentiation and activation of macrophages.^{77,78} Wang *et al.* found that macrophages and invasive tumour cells migrate together, but only in response to CSF-1 and epidermal growth factor (EGF).⁷⁹

The initial phosphorylation of the CSF-1R tyrosine residues could be prevented by blocking the CSF-1R kinase which would prevent macrophage development, proliferation and differentiation. CSF-1R is highly expressed in macrophages so the delivery of small molecule tyrosine kinase inhibitors, such as Dasatinib (**Figure 7**), to tumour associated macrophages has the potential to be an effective cancer treatment. Selective delivery to macrophages would mean that any side-effects were minimal as the drug would not be internalised by other cell types. Selective delivery also allows for the use of lower drug doses as a much larger

percentage of the administered drug will reach target cells compared to when systemic treatment methods are used.

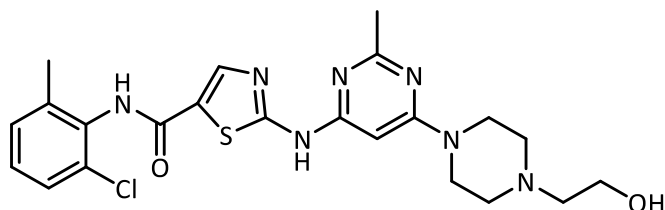


Figure 7. Dasatinib, a tyrosine kinase inhibitor.

1.2.2 Targeted drug delivery to macrophages

There are two main types of targeted drug delivery, passive targeting and active targeting.^{17,80} Passive targeting takes advantage of differences in the microvasculature structure of different types of tissue. For example, tumour cells are often surrounded by leaky microvasculature which means that any drugs or delivery systems administered to the area will get increased admittance to tumour tissue in comparison to healthy tissue. This is known as the enhanced permeability and retention (EPR) effect.⁸¹ Inflamed tissue is also known to have leaky microvasculature. Active targeting involves designing delivery systems which interact with the target cell but that will not interact with other cells in the body. The 'Trojan horse' approach is one example of active targeting; drugs can be attached to molecules known to be required, and therefore taken up, by some cells but not by others. An example of this is polyamines; polyamines are needed for all cell proliferation in the body.^{82,83} Cancer cells proliferate rapidly and cannot synthesise the required amounts of polyamines to sustain their proliferation. To increase their polyamine levels, cancer cells internalise the compounds from the extracellular medium around them.⁸⁴ Polyamines enter the cells *via* the polyamine transport system which is enhanced in cancer cells and can be used to selectively deliver chemotherapeutic agents to cancer cells but not to healthy cells.⁸⁵ Another example of active targeting is using receptors that are either over expressed, or only ever found on the target cell. Ligands that selectively bind to certain receptors can be attached to drug delivery systems. This type of targeted delivery works best for systems which standardly have very low uptake and need help to be internalised by the target cell. For delivery systems which have high levels of cellular uptake the receptor targeted approach has considerably less use; designing a drug delivery system

which has increased uptake by the target cell will not prevent its uptake by other cells in the body.

There are many factors which are known to influence a particle's uptake by macrophages, in particular, size, surface charge and surface ligands.³⁹ However, there is still much mystery around selectively targeting macrophages. Research groups who have succeeded in delivering cargo selectively to macrophages often don't have an explanation for why or how their particular delivery system is selective. Wang *et al.* were able to selectively target intestinal macrophages using naturally occurring nanovesicles derived from grapefruit.² Wang *et al.* found that, when administered orally, their nanovesicles accumulated in the large intestine and that the majority of the vesicles had been internalised by macrophages. *In vitro* studies suggested that the particles were internalised *via* a mixture of macropinocytosis and clathrin-dependent mechanisms. However, many cells in the body are capable of macropinocytosis and clathrin-mediated endocytosis so the mechanism of the selective delivery of these particles to macrophages is unclear.²

Much talk of 'selectivity' concerns targeting specific macrophage receptors, a number of macrophage receptors have been identified. The macrophage mannose receptor is one of the most commonly targeted for selective delivery purposes.^{38,86-88} As discussed in section **1.2**, the mannose receptor binds to mannose and fucose sugars and is found in abundance on macrophages.⁸⁹ The Fc receptor, a protein located on a number of cells of the immune system which binds to antibodies found on pathogens, is also a popular target.^{72,90} Other macrophage surface receptors include Dectin-1.⁹¹ Dectin-1 is a C-type lectin-like receptor which is also found on monocytes, neutrophils, dendritic cells and some T-cells. Systems which have very low levels of internalisation *in vivo* can be selectively targeted to macrophages using these receptors. For example, Liang *et al.* used a substituted mannosylated poly(L-lysine) to target the delivery of antisense oligonucleotides to the macrophage mannose receptor. Without the attachment of the mannosylated poly(L-lysine), the oligonucleotides had inefficient cellular uptake, this made selective macrophage targeting *via* the mannose receptor pathway possible.⁸⁷ Delivery systems with high levels of cellular uptake have also been decorated with mannose. For example, Asthana *et al.* determined that chitosan nanocapsules coated with mannose were internalised significantly more by macrophages than uncoated nanocapsules.⁸⁸ It should be noted, however, that as the uncoated nanocapsules were still

internalised, it is unlikely that delivery to macrophages was selective. Many other groups have had similar findings.^{92–95} While targeting the mannose receptor (or other macrophage receptors) will increase uptake by macrophages, it will not decrease uptake by other cell types.

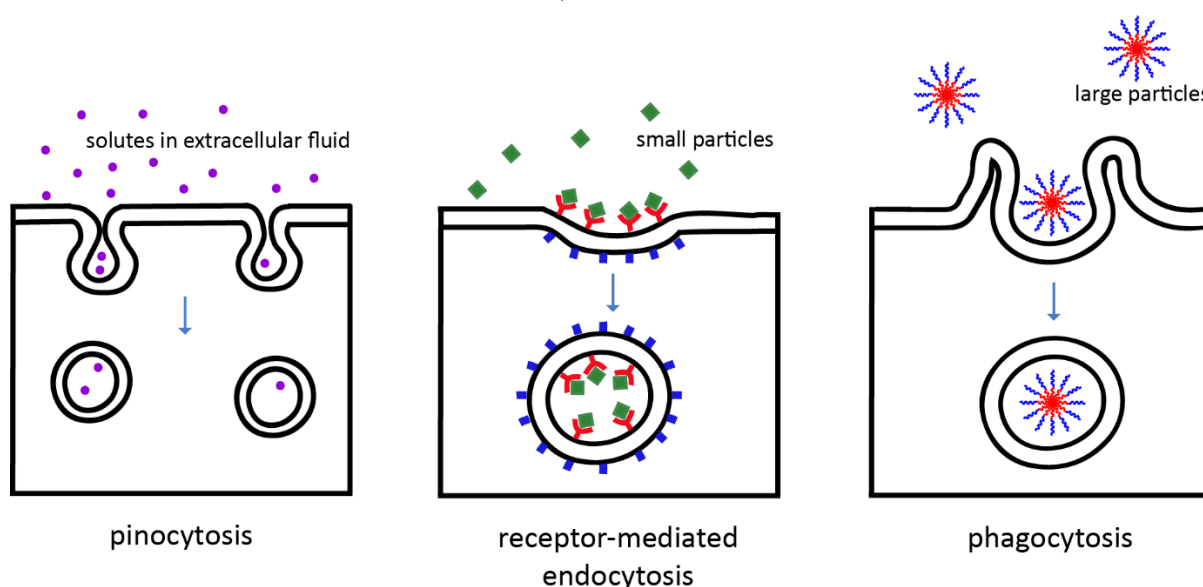


Figure 8. Uptake mechanisms, pinocytosis, receptor mediated endocytosis and phagocytosis. Large particles (approximately 500–750 nm) can be phagocytosed but not internalised via other endocytosis mechanisms.

The majority of drug delivery systems currently designed to selectively target macrophages show proof of efficient delivery but less focus has been centred on preventing uptake by other cell types. One way to ensure selective uptake by macrophages is to target the phagocytic pathway. Macrophages are one of few cell types in the body which are able to phagocytose. If a delivery system was engineered to be phagocytosed but not taken up *via* other endocytosis, or passive uptake mechanisms then selective macrophage delivery could be achieved. Phagocytosis involves the internalisation of much larger particles than other types of endocytosis (**Figure 8**), making size an obvious choice for engineering targeted delivery to phagocytic cells such as macrophages. Literature findings concerning the size cut-off point for selective delivery to macrophages are not in complete agreement. Hoekstra *et al.* found that non-phagocytic B16 cells internalised latex beads up to 500 nm when a range in beads sized from 50–1000 nm was tested, indicating that the cut-off for non-phagocytic uptake is 500 nm.⁹⁶ However, Koval *et al.* studied the uptake of IgG-opsonized polystyrene beads by murine primary bone marrow macrophages and found that beads with a diameter ≤ 750 nm

were internalised *via* receptor mediated endocytosis whereas beads > 750 nm were internalised *via* phagocytosis.⁹⁷ Koval's findings indicate that the cut-off size for non-phagocytic uptake is 750 nm. The books 'Molecular regulation of endocytosis' by Brian Ceresa and 'Nanoparticulate Drug Delivery Systems: Strategies, Technologies, and applications' by Yoon Yeo both quote the size cut-off for particle uptake *via* non-phagocytic mechanisms to be 500 nm.^{63,98}

1.3 Controlled Radical Polymerisation

Radical polymerisation is used to make nearly 50% of all commercial polymers.⁹⁹ In conventional radical polymerisation termination is a frequent occurrence and the reaction rate is diffusion controlled.¹⁰⁰ Controlled, or 'living' radical polymerisation allows for regulation over aspects of the synthesised polymer such as the molecular weight, the polydispersity, the composition of the polymer and the functionality of the polymer. In a controlled radical polymerisation reaction, the polymer's molecular weight is increased in a linear fashion with time and occurrences of early termination are reduced compared to in uncontrolled polymerisation. There are three main types of controlled radical polymerisation, atom transfer radical polymerisation (ATRP), nitroxide-mediated polymerisation (NMP) and reversible addition/fragmentation chain transfer polymerisation (RAFT).^{100–103} Controlled free radical polymerisation methods work by extending the lifetime of propagating radical polymer chains so that radical–radical termination is avoided. To extend the lifetime of the propagating radicals a dynamic equilibrium is used: propagating radicals are constantly equilibrating between the active propagating chains and dormant chains which cannot terminate. By keeping the concentration of the active propagating chains low and the dormant chains high, terminations are avoided.¹⁰⁰ The different methods of controlled polymerisation vary in terms of how a dynamic equilibrium is achieved.^{104–106}

In ATRP a metal complex catalyst is used to control the equilibrium.¹⁰⁷ An alkyl halide initiates the reaction. The halide can either be coordinated to the metal centre or attached to the propagating polymer chain to give a halogen-terminated dormant polymer chain. Re-initiation of the polymerisation occurs *via* reaction with the metal catalyst to give the halogen bound to the metal catalyst and the polymer as an active propagating radical

(**Figure 9**). This equilibrium between dormant polymer chain and active polymer chain is what provides the control over the polymerisation.¹⁰⁷

In NMP the initiator reacts with a monomer to give a propagating radical, this can either react with another monomer or with a nitroxide radical which traps the propagating radical species.¹⁰⁸ Reaction with the nitroxide radical is reversible so an equilibrium is formed between the trapped and active propagating radical species (**Figure 9**), as in ATRP, it is this equilibrium between dormant (or trapped) and active propagating radicals that provides the control in the chain growth to give low dispersity polymers.¹⁰⁸

In RAFT polymerisation an equilibrium exists between the propagating radical polymer chain and the dormant polymer chain attached to the RAFT agent.¹⁰¹ The RAFT mechanism is discussed further in the following section (**1.3.1**).

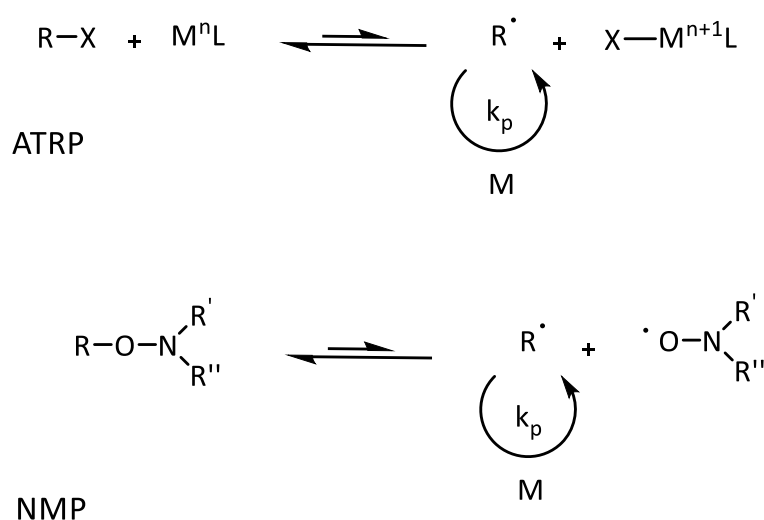


Figure 9. Equilibration mechanisms for ATRP (top) and NMP (below).

There are advantages and disadvantages of each controlled radical polymerisation technique. ATRP and RAFT are both very versatile and can be tailored to specific reaction needs and conditions.¹⁰⁹ Disadvantages of ATRP include the use of transition metals and the need for high reaction temperatures. A disadvantage to RAFT polymerisation is the odour that is often produced by the reactions. NMP polymerisation does not involve the use of transition metals or have a high capacity to produce odour but it is less versatile than either ATRP or RAFT as it is limited to certain solvents and monomer functionalities.¹⁰⁹

Using block copolymers synthesised *via* controlled radical polymerisation techniques to make polymeric structures, such as micelles, gives high control over micelle composition due to the manipulability of the techniques.^{100,101} The polymer composition can be altered to adapt the properties of the micelle meaning they can be tailored to suit a wide range of applications.^{110,111}

1.3.1 RAFT polymerisation

This project is a collaboration between Imperial College London and CSIRO. CSIRO initially developed the technique of RAFT polymerisation in 1998 and their expertise on the subject and on experimental techniques has been invaluable to the project.¹⁰¹

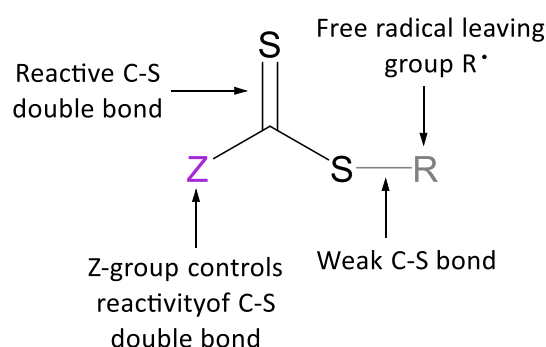


Figure 10. Structure of RAFT agent and how different groups affect reactivity.¹⁰⁴

RAFT polymerisation is a non-specific polymerisation process which produces controlled molecular weight polymers using thiocarbonylthio compounds (**Figure 10**).¹⁰⁴ RAFT polymerisation allows for the controlled synthesis of a huge range of block copolymers with low polydispersities (**Figure 11**).^{112,113} The polydispersity of RAFT synthesised polymers is standardly < 1.2 and often < 1.1 .¹⁰¹ Since the development of the technique in 1998, RAFT polymerisation has been used to make a huge variety of polymeric structures such as micelles, graft block copolymers, honeycomb-structured porous films, microgels and star polymers.^{112,114–121}

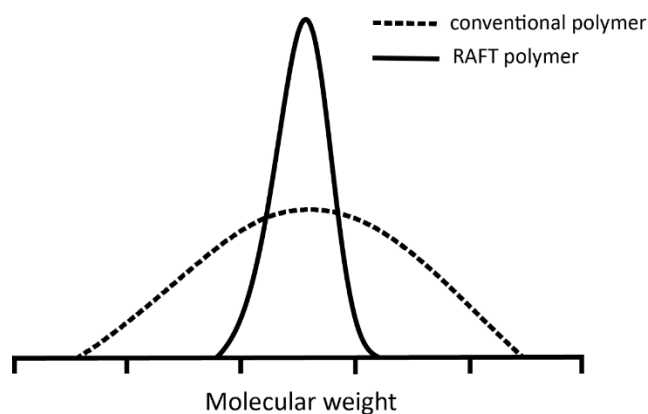
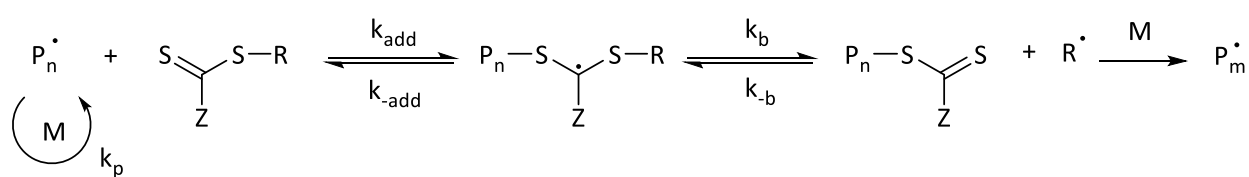


Figure 11. Polydispersity of a polymer synthesised via RAFT polymerisation compared to a polymer synthesised via conventional polymerisation.

The RAFT polymerisation reaction (**Figure 12**) begins with initiation, to give the propagating radical ($P_n^\bullet$). The propagating radical is then added to the thiocarbonylthio compound [$P_nS(Z)C=S$] to produce a new radical ($R^\bullet$). The new radical then attacks the monomer to form a new propagating radical ($P_m^\bullet$). The equilibrium between the two propagating radicals ($P_n^\bullet$ and $P_m^\bullet$) and the polymeric thiocarbonylthio compounds means that there is an equal probability for all of the chains to be extended and it is because of this equal probability that there is such a narrow poly dispersity in the polymers produced. Once the polymerisation reaction is complete the thiocarbonylthio end group remains attached to the polymer chains which can then be isolated.

Reversible chain transfer/ propagation



Chain equilibration/ propagation

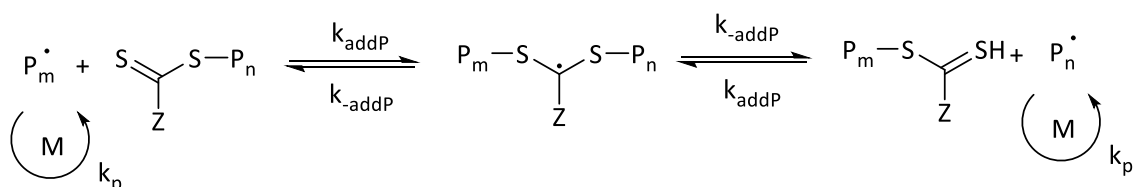


Figure 12. Mechanism of RAFT polymerisation.¹⁰¹

The main advantage of RAFT over other controlled polymerisation techniques is the flexibility of the method. There are no limitations on solvents or reaction temperatures and a wide range of monomers, including monomers with acid, tertiary amino and hydroxyl functionality, can be used.¹⁰¹ The wide scope of monomers and reaction conditions means that RAFT polymerisation has a huge range of applications.^{122,123} Over 50 companies ranging from DuPont to L'Oréal have filed patents relating to the use of RAFT polymerisation for various products.¹²⁴ Applications of RAFT include optoelectronic polymers, polymer bioconjugates, stimuli responsive drug delivery systems, the surface modification of nanoparticles, paper coating and weather resistant coatings.^{125–139}

RAFT drug delivery systems

RAFT has been utilised in the synthesis of a wide number of drug delivery systems.^{111,140,141} Micelles, polymersomes and star polymers are all common delivery vehicles that can be synthesised using RAFT polymerisation (**Figure 13**).¹⁴² Micelles are self-assembled from amphiphilic block copolymers under aqueous conditions; the hydrophobic section of the polymer is internalised to reduce unfavourable interactions with water, while the micelle shell is hydrophilic.¹⁰⁴ Polymersomes are also made from amphiphilic block copolymers in aqueous solutions but form vesicle-like structures instead of micelles particles to internalise the hydrophobic section of the polymer.¹²² Star polymers are commonly grown from a multifunctional core (core-first approach) but can also be synthesised first as polymer chains and then assembled *via* reaction with the multifunctional core (arm-first approach).¹⁴²

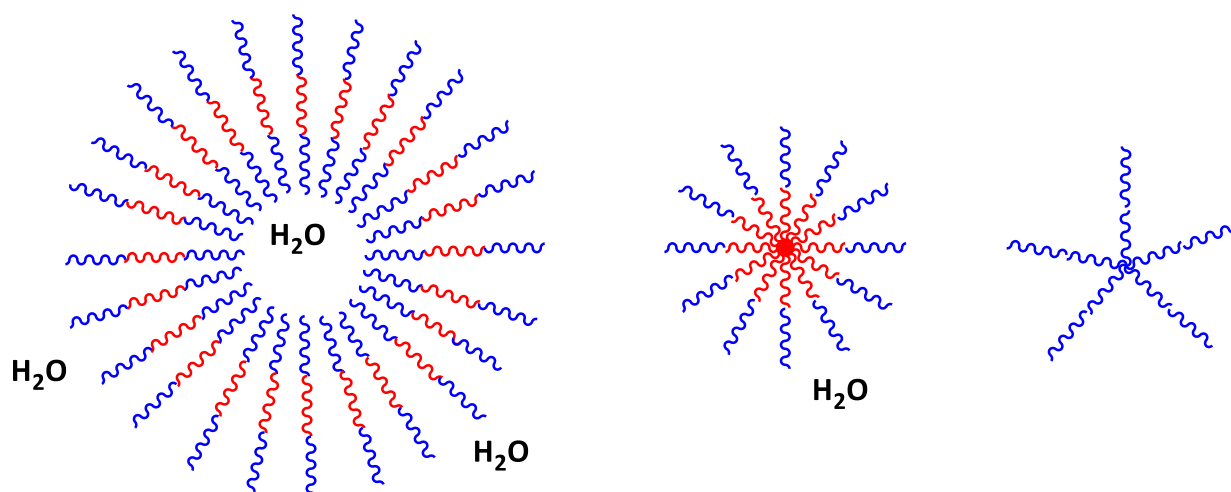


Figure 13. From left to right; polymersomes, micelles and star polymers can all be synthesised *via* RAFT for use as drug delivery vehicles.

RAFT micelles for drug delivery have been synthesised by a large number of groups. Well-known examples include work by Stenzel *et al.*, who have recently designed and tested block copolymers which react with the chemotherapeutic $\text{RuCl}_2(\text{p-cymene})$ to give a micellular drug with increased uptake and anti-tumour properties compared to that of the free drug.^{143,144} One of the most famous micelle drug delivery systems is Kayaku *et al.*'s doxorubicin delivery particle, NK911, discussed in 1.1.1, which has reached clinical trials in Japan.²⁶

Zhong *et al.* used RAFT to synthesise pH-sensitive polymersomes for the delivery of doxorubicin to HeLa cells.¹⁴⁵ Another example of RAFT polymersomes encapsulating doxorubicin is Kharlampieva *et al.*'s synthesis of polymersomes, made from triblock copolymers $\text{poly}(N\text{-vinylcaprolactam})_n\text{-poly}(\text{dimethylsiloxane})_{65}\text{-poly}(\text{vinylcaprolactam})_n$.¹⁴⁶

Examples of RAFT star polymers include work by Boyer *et al.*, in which poly(ethylene glycol) methyl ether acrylate polymers were synthesised and then chain extended using vinyl benzaldehyde and the reversible crosslinker, N,N' -bis(acryloyl)cystamine.¹⁴⁷ Boyer *et al.* were able to incorporate doxorubin using the aldehyde functionality in the core of the particles, which were successfully internalised by cells. Thang *et al.* took a different approach, making mikto-arm star polymers with three different types of polymer arms.¹⁴⁸ One arm was hydrophobic (butyl methacrylate based), one was cationizable (dimethylaminoethyl methacrylate based) and one was hydrophilic (poly(ethylene glycol) based). The anti-cancer drug 7-ethyl-10-hydroxycamptothecin (SN-38) was incorporated into each polymer arm.

Table 1 outlines further examples of successful RAFT drug delivery systems developed in the past twelve years. Micelles have been more extensively used as delivery systems than either polymersomes or star polymers.¹⁴⁹

Research group	Delivery vehicle	Polymer	Cargo	Cell type
Yamauchi <i>et al.</i> ¹⁵⁰	Micelle	Poly(styrene- <i>b</i> -acrylic acid- <i>b</i> -ethylene glycol)	Doxorubicin	BT-20 human breast cancer cells
Stenzel <i>et al.</i> ¹⁵¹	Micelle	Poly(polyethylene glycol methyl ether methacrylate-co-methacrylic acid)- <i>b</i> -poly(methyl methacrylate)	Albendazole	Human prostate cancer cells (PC-3, DU-145) and human ovarian cancer cells (OVCAR-3, A-2780)
Stayton <i>et al.</i> ¹⁵²	Micelle	Poly(ethylene glycol)- <i>b</i> -diethylaminoethyl methacrylate-co-butyl methacrylate	Bcl-2 interacting mediator peptide drug	<i>In vivo</i> - Ramos tumour-xenograft mice Cell lines- Ramos AW, Ramos, Jeko-1, Daudi, DOHH2, Raji, JVM-2 Granta 519, JVM-13, K562
Zentel <i>et al.</i> ¹⁵³	Polymersome	Poly(<i>N</i> -(2-hydroxypropyl) methacrylamide)- <i>b</i> -poly(dodecyl methacrylate)	Sulforhodamine B	MTT (colorimetric) assay
Kissel <i>et al.</i> ¹⁵⁴	Micelle	Poly(ϵ -caprolactone)- <i>b</i> -poly(ethylene glycol)	Paclitaxel	NIH/3T3 (Swiss mouse embryo) cell line
Zhong <i>et al.</i> ¹⁵⁵	Polymersome	Poly(ethylene glycol)-SS-poly(2-(diethyl amino) ethyl methacrylate)	FITC-labelled bovine serum albumin and FITC-labelled cytochrome C	RAW 264.7 and MCF-7 cell lines
Kavallaris <i>et al.</i> ¹⁵⁶	Star polymer	Poly(<i>n</i> -butyl acrylate)- <i>g</i> -poly(ethylene glycol) acrylate	BODIPY (boron-dipyrromethene)	Human neuroblastoma (SH-EP) cells

Table 1. RAFT delivery systems.

1.4 Project aims

The aim of this project is to synthesise a range of polymeric micelle particles *via* RAFT polymerisation, for selective drug delivery to macrophages (**Figure 14**). It is our hypothesis that macrophages can be targeted using micelles large enough to be selectively phagocytosed. The synthetic micelles need to be able to encapsulate small drug molecules and also have to have the right properties to ensure they are successfully internalised by macrophages. A fluorescent tag has to be incorporated into the micelles so that the particles can be tracked *in vitro* and *in vivo*. The design and synthesis of a range of polymeric micelles is discussed in **Chapter II**. **Chapter III** of this thesis concerns the adaptation of micelles in preparation for cellular uptake studies. In **Chapter IV** the biological uptake of different micelles by macrophage and non-macrophage cells is discussed, alongside the potential for micelles to successfully encapsulate and deliver a fluorescent cargo.

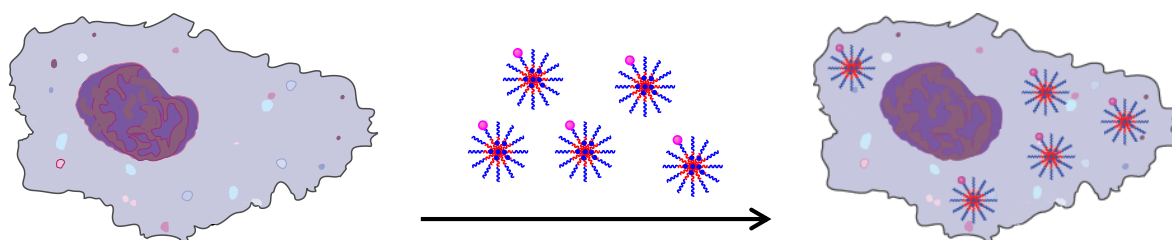


Figure 14. Internalisation of fluorescently labelled, drug loaded micelles by a macrophage.

Chapter II

Synthesis of Polymeric Micelles

2.1 Introduction

2.1.1 Micelles

The formation of micelle particles from amphiphilic block copolymers has been well studied since micelles were first discovered to have potential for drug delivery in the 1980s.¹⁵⁷ Block copolymers are polymer chains which have blocks made of different monomers. One, or multiple blocks can be either linear or branched.¹⁵⁸ The properties of block copolymers are defined by their structure and composition.¹⁵⁹ For example, longer polymer chains have higher melting and boiling points. The strength and impact resistance of a polymer, or material made from a polymer, also increase with increasing chain length.^{159,160} Branched polymers form much more viscous solutions than linear polymers.¹⁶¹ The hydrophobicity of the polymer affects how soluble it is in different solvents.¹⁵⁹ A great amount of control is afforded over block copolymers and their properties as their composition can be easily manipulated, making them extremely versatile and adaptable. As well as micelle formation, block copolymers have a wide range of uses; polymersomes, lamellae and surface patterning are common applications.^{162–164}

As outlined in **Chapter I**, a number of successful micelle delivery systems have been created. Drugs, DNA and fluorescent dyes have all been incorporated into micelles for drug delivery. The anti-cancer drug, doxorubicin, has been encapsulated in a number of micellar systems including the poly(ethylene glycol)-*b*-poly(aspartic acid) block copolymer micelles synthesised by Kayaku *et al.* (known as NK-911) which were successful in phase I clinical trials in Japan, a more detailed description of this system can be found in section **1.1.1**.^{25,26} For micelle formation to occur polymers need to have a hydrophilic and a hydrophobic portion. The hydrophobic portion is internalised under physiological conditions, resulting in a self-assembled micelle particle which can encapsulate small drug molecules (**Figure 15**).^{165–169}

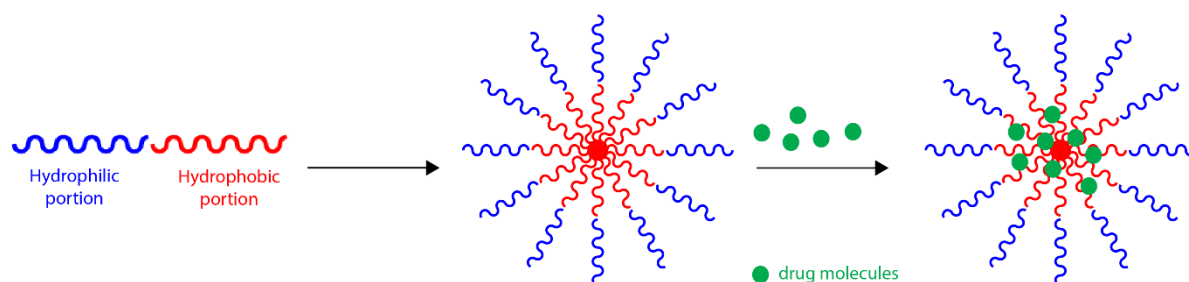


Figure 15. Drug encapsulation in hydrophobic core of micelle particle.

For block copolymers to form micelles in solution, the concentration of the solution must be above the critical micelle concentration (CMC).¹⁷⁰ At low concentrations the hydrophobic portion of the chain adsorbs at the water-air interface to reduce hydrophobic-water interactions.¹⁷¹ As the concentration is increased, the interface reaches full capacity and it is at this point (the CMC) that micelles start to form. Micelles only exist above the CMC and will usually disassemble into unimers if the concentration is reduced below the CMC.¹⁷¹

Experimental studies using micelles as drug delivery agents have shown that micelle particles often need to be stabilised with cross-linkers before they can be used *in vivo* as they are diluted to very low concentrations once administered to patients.^{172–174} Micelles can be crosslinked by connecting adjacent micellar polymer chains together with a linker (usually a difunctionalised hydrocarbon chain), this prevents the micelles from being able to disassemble. Studies have shown that, except for enhancing the stability of the micelle, crosslinking generally has a minimal impact on the structure of the particle.^{175,176} Different crosslinking methodologies are described in **Chapter III** in section **3.1.1**.

When block copolymers are dissolved in a solvent there are three different processes that can occur, the polymers can either exist as unimers, form individual micelles or form a gel. Micelles form when the polymers are dissolved in a large volume of polar solvent (providing the block copolymer concentration is above the CMC). The polar section of the block copolymer remains ‘facing outwards’ and interacts with the solvent while the hydrophobic portion of the block copolymer is internalised within the micelle to be shielded from the solvent. When the concentration of block copolymer is high the micelles become ordered and form a gel.¹⁷⁰

2.1.2 Effect of polymer composition on micelle

Research into the effect of polymer chain length on micelle formation has found that longer polymer chains generally form larger micelle particles.^{177,178} Stolnik *et al.* studied nanoparticles formed from poly(lactic acid)-poly(ethylene glycol) block copolymers and determined that the hydrophobic (poly(lactic acid)) block molecular weight correlated with micelle size.^{179,180} Lavasanifar *et al.* also found that increasing the hydrophobic block on their poly(ethylene oxide)-*block*-poly(hydroxyl alkyl L-aspartamide) block copolymer resulted in the formation of larger micelles.¹⁸¹

The ratio of hydrophilic to hydrophobic block molecular weight has an influence on the solubility of the polymer chains in water and therefore has an effect on micelle formation. Polymers with larger hydrophilic blocks are easily soluble in water. Polymers with larger hydrophobic blocks are less soluble, due to the reduced attractive forces between the polymer and water. This can lead to difficulties in terms of micelle self-assembly; if the polymer is only sparingly soluble in water then the CMC may not be reached and micelles will not form.¹⁶² The ratio of hydrophilic to hydrophobic block molecular weight also has an effect on the shape of the self-assembled structure.¹⁸² Eisenberg *et al.* synthesised 'crew cut' micelle-like aggregates and found that as the hydrophilic section of their polymers was reduced the structures changed from small micelles (26 nm), to rod-like micelles, to vesicular structures, to large complex micelles (up to 1.2 μm) containing small reverse micelles (**Figure 16**).¹⁸³ Providing that the polymer is soluble, the more hydrophobic the core of the micelle is (i.e. if more hydrophobic monomers are used) the more stable the micelles will be and the lower the CMC.^{177,182,184}

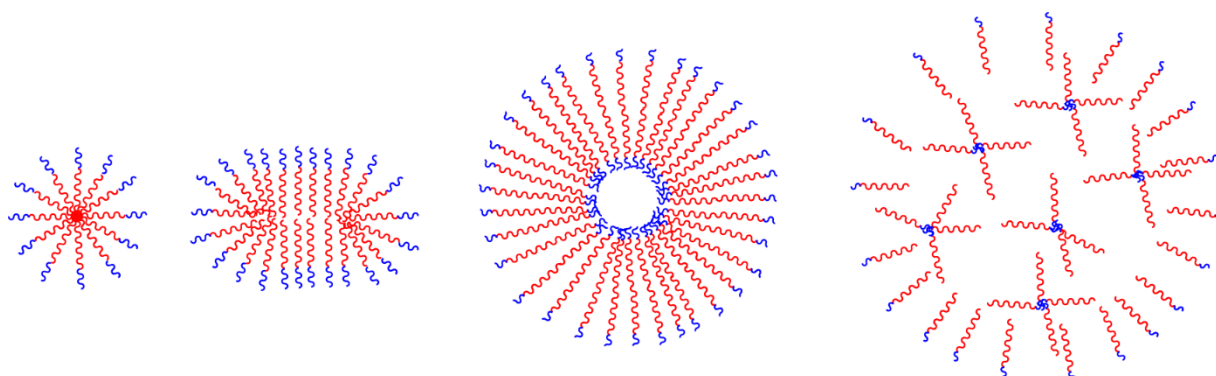


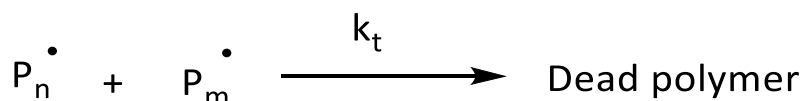
Figure 16. From left to right: 'crew cut' micelle, rod-like micelle, vesicular structures and large complex micelles containing small reverse micelles.¹⁸³

2.1.3 Selecting a RAFT agent

A large number of RAFT agents have been synthesised and used in various polymerisation reactions including dithiobenzoates, xanthates, dithiocarbamates and trithiocarbonates.¹⁰⁴ The monomer(s) and reaction conditions used in a reaction both have an effect on how well the RAFT agent performs. It is important to select a RAFT agent with the right reactivity for your chosen system or some of the advantages of RAFT polymerisation, such as the low polydispersity of the polymers formed, can be lost.¹⁰⁴ Different RAFT agents have different rates of addition and fragmentation (see section 1.3.1 for RAFT mechanism).

The R and Z group both control the reactivity of the RAFT agent (**Figure 17**). The weaker the S—R bond, the easier the free radical leaving group R[•] is formed. It is important that the free radical R[•] is a better leaving group than the attacking propagating radical. For example, in the polymerisation of monomers such as methacrylates R should not be an aromatic as the methacrylyl radical will be a better leaving group than R[•]. R[•] also plays a role in re-initiation so it is important that it is able to re-instigate the polymerisation. The Z group controls the reactivity of the C=S double bond, with different Z groups able to either activate or deactivate the bond. The reactivity of the C=S double bond affects the rate of addition of propagating radicals; the more reactive the bond the faster the rate of addition. In polymerisations of methacrylate monomers, the Z group should be an aryl, S-alkyl or a pyrrole and the R group should be a tertiary cyanoalkyl or cumyl group.¹⁰⁴ More generally, if acrylates, acrylamides or styrenic monomers are being polymerised then a tertiary cyanoalkyl trithiocarbonate RAFT agent will work well and if vinyl monomers are being polymerised then a cyanoalkyl xanthate is a good option.¹⁰⁴

Termination



Scheme 2. Two initiated radicals reacting together to form a dead polymer chain which is unable to extend via RAFT polymerisation.¹¹²

2.1.5 Quasi-block copolymers

In the synthesis of block copolymers a lengthy purification is required after each polymerisation to remove left over monomer, initiator and the reaction solvent. This makes it difficult to synthesise polymers efficiently; the synthesis of a simple diblock copolymer can take over five days.

To overcome these limitations one-pot polymerisation techniques have been developed.^{119,186,187} When monomers have large differences in reactivity, one monomer will polymerise preferentially over the other. The second monomer will not start to polymerise until the majority of the more reactive monomer has been converted to polymer. This leads to the formation of two polymer blocks with a gradient between them, these are known as gradient block copolymers.^{188,189} This method significantly reduces overall synthesis time as only one purification step has to be carried out at the end of the synthesis. However, although this technique provides a quicker route to block copolymers, it can only be used for systems where there is a large reactivity ratio between monomers. For the majority of monomer combinations, a random copolymer would be formed. For applications such as micelle self-assembly, it is essential for the two blocks to be well defined as the self-assembly step relies on the difference in hydrophobicity of the two polymer blocks.

Quasi-block copolymerisation is a promising alternative that allows for the synthesis of gradient block copolymers when monomers have similar reactivity. Quasi-block copolymers are formed *via* sequential RAFT polymerisations in one pot. The initial polymerisation reaction is carried out as normal but instead of purifying the polymer before the block copolymerisation, the second monomer is added to the reaction mixture. The polymers formed are likely to be gradient block copolymers, as opposed to pure-block copolymers (**Figure 18**); if low levels of conversion are reached in the first step of the reaction there is a

high likelihood of there being a monomer impurity in the second block.¹⁹⁰ Ideally, the first step of the reaction should be run until 100% monomer conversion has been reached. This would prevent contamination of the second block, however, dead chain formation is a common problem when monomer conversions > 80% are reached.¹⁹⁰ Quasi-block copolymer synthesis has obvious advantages over pure-block copolymer synthesis; without the lengthy copolymer purification steps block copolymers can be synthesised much more efficiently and at a lower cost.



Figure 18. Pure-block copolymer (left) and quasi-block copolymer (right). Small amounts of left over monomer from the first step of the reaction become part of the second block in the quasi-block copolymer.

Quasi-block copolymers are cheaper and much less time consuming to make than pure-block copolymers so synthesizing polymer libraries using the quasi-block method is a vast improvement to using the standard method for pure-block copolymers. In an experimental study Guerrero-Sanchez *et al.* synthesised a library of quasi-block copolymers which were compared to the equivalent pure-block copolymers using differential scanning calorimetry.¹⁹⁰ Guerrero-Sanchez *et al.* determined that although quasi-block copolymers had a slightly different composition to pure-block copolymers (as some of the left-over monomers from the copolymerisation reaction polymerise to become part of the second block) the properties, as measured by differential scanning calorimetry, of the two different types of block copolymer were very similar.¹⁹⁰ Schubert *et al.* also synthesised and compared a range of quasi and pure-block copolymers (poly[2-(dimethylamino)ethyl methacrylate-*b*-di(ethylene glycol)methyl ether methacrylate]). No difference was seen between the turbidity measurements of the pure and quasi-block copolymers.¹⁸⁶

Initial research in the area of quasi-block copolymers, showing them to have very similar properties to pure-block copolymers, suggested that micelle formation would still be possible with quasi-block copolymers. However, as previously discussed, self-assembly into micelles requires distinct hydrophobic and hydrophilic blocks. The presence of hydrophobic monomer units in the hydrophilic polymer block could disrupt micelle formation. Thang *et al.* synthesised pure and quasi poly(ethylene glycol)methyl ether methacrylate-*b*-*N,N*-

dimethylaminoethyl methacrylate-*b*-poly(ethylene glycol) methyl ether methacrylate triblock copolymers for siRNA delivery.¹⁹¹ The two polymer types had different delivery efficacies; quasi-triblock copolymers were able to deliver active siRNA more efficiently than pure triblock copolymers. In summary, research to date indicates that quasi-block copolymers have similar properties to pure-block copolymers but that small differences in polymer composition can have a large effect on the structures assembled from them, and therefore on the biological properties of the polymer.

In this chapter the synthesis of a library of polymeric micelles is discussed. The aim was to synthesise a library of polymeric micelles ranging in size from 20–500 nm for drug delivery to macrophages. As discussed in **Chapter I**, section **1.2.1**, numerous diseases require the delivery of drugs to macrophages to ensure efficacious treatment.^{53–68} As discussed in section **1.2.2**, nanoparticles can be selectively targeted to macrophages using size.³⁹ Due to the lengthy synthesis time required for block copolymers, the synthesis of quasi-block copolymers was also investigated and their potential use in this project evaluated. It was hypothesised that changing the length and composition of polymer chains would influence the size of the assembled micelle with higher ratios of MAA to BMA and longer polymer chains resulting in larger micelles. With regards to quasi-block micelle formation it was predicted that quasi-block copolymers would form similar micelles to those formed from pure-block copolymers.

2.1.6 Chemspeed

The use of automated parallel synthesisers, such as the Chemspeed range, has become common when creating large polymer libraries *via* RAFT polymerisation and other controlled radical polymerisation techniques.¹⁹² Parallel synthesisers have hugely improved the efficiency of polymer synthesis as multiple reactions can be run simultaneously and largely unattended.^{193,194} Results have been proven to be reproducible both within and between synthesisers.¹⁹⁵

2.1.7 Micelle design

The hydrophobic portion of the polymer was planned to be made up of butyl methacrylate (BMA) and methacrylic acid (MAA) in ratios of; 9: 1, 4: 1, 2: 1 (BMA: MAA). The polar portion

MAA BMA PEGMA

Hydrophobic tail Polar head group

Poly(ethylene glycol) (PEG) is commonly used in drug delivery systems as it can extend circulation times due to its high hydrophilicity and is safe for *in vivo* use.^{21,111,141,196} Circulation times of PEG coated particles are extended as its hydrophilicity means that the polymer is highly hydrated *in vivo*. This hydration prevents the adsorption of opsonin, which is the first step in the clearance of a particle. PEGMA was used to ensure compatibility with the RAFT agent used in the polymerisation of BMA and MAA.¹⁹⁷ MAA was selected to give the micelle core acidic functionality to stabilise the encapsulation of a basic drug (e.g. Dasatinib, discussed in **1.2.1**) or dye. BMA was selected alongside MAA as the monomers are both methacrylates and are known to be polymerised at a similar rate.^{101,198} The rate of polymerisation of two monomers in a copolymer dictates if the monomers are evenly distributed through the polymer chain or if a gradient copolymer is formed.¹⁰⁴ The planned ratio of monomers in the hydrophobic portion of the polymer was altered to potentially give micelle particles of different sizes. Size has been determined by experimental studies to be the most influential factor on efficiency of uptake by macrophages.^{39,199}

Preliminary work on this project was carried out by Dr R Davidson during a placement at CSIRO, Melbourne, Australia. BMA-co-MAA copolymers were synthesised using two different RAFT agents (**Figure 20**). RAFT agent **1** is recommended for use with methacrylate systems.¹⁰⁴ RAFT agent **2** was investigated as when this RAFT agent is used it is possible to calculate polymer molecular weight accurately from ¹H NMR spectra. This is done by comparing the RAFT agent aromatic peaks' (δ 7.25–8.00) integrals to polymer peak integrals.

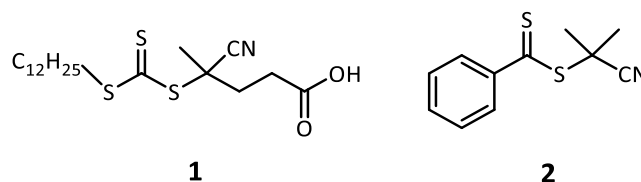


Figure 20. RAFT agents used to synthesise BMA-co-MAA copolymers: 4-cyano-4[(dodecylsulfenylthio carbonyl)sulfanyl] pentanoic acid (left) and 2-cyano-2-propyl benzodithioate (right).

The results are displayed in **Table 2**; polymers synthesised using dithiobenzoate RAFT agent **2** required longer reaction times which lead to the occurrence of more side reactions, indicated by higher dispersity ($\mathcal{D}_M$) values. The use of RAFT agent **2** resulted in the synthesis of polymers with a much higher polydispersity, 1.23–1.50, than those synthesised using RAFT agent **1**, ≤ 1.10 . RAFT agent **1** was therefore determined to be more suitable and was used in further reactions.

RAFT agent	BMA: MAA	M_n (Da)	$\mathcal{D}_M$	% yield
1	9: 1	5,200	1.08	70
1	4: 1	4,900	1.08	75
1	2: 1	4,100	1.10	75
2	9: 1	6,500	1.23	45
2	4: 1	5,700	1.25	45
2	2: 1	5,700	1.50	70

Table 2. Comparison of BMA-co-MAA copolymers synthesised using RAFT agent 1 and 2. M_n is the number averaged molecular weight, $\mathcal{D}_M$ is the molecular weight dispersity ($= M_w/M_n$). M_w is weight averaged molecular weight. M_n and $\mathcal{D}_M$ were determined via gel permeation chromatography (GPC, THF SEC).

BMA-co-MAA copolymers synthesised using RAFT agent 1 were chain extended to BMA-co-MAA-*b*-PEGMA block copolymers which were self-assembled into micelles. Polymers with a higher percentage of MAA in the hydrophobic block formed larger micelles (**Table 3**).

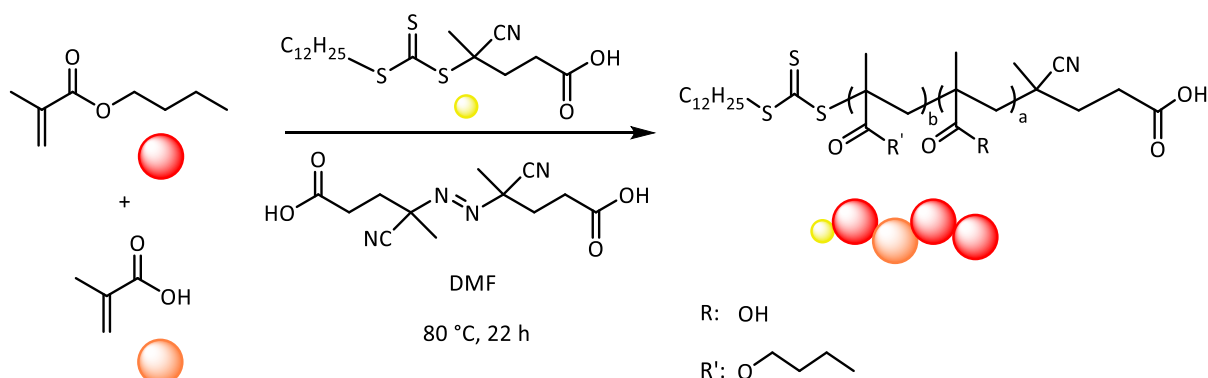
BMA: MAA	Target MW of BMA-co-MAA block (Da)	Target MW of PEGMA block (Da)	M_n (Da)	$\mathcal{D}_M$	Micelle diameter (nm)
9: 1	5,000	5,000	7,400	1.18	45
4: 1	5,000	5,000	7,100	1.30	110
2: 1	5,000	5,000	5,800	1.26	300

Table 3. BMA-co-MAA-*b*-PEGMA block copolymer molecular weights and dispersity alongside the size of micelles formed from the self-assembly of the block copolymers. Micelle diameter measured using DLS.

2.2 Results and Discussion

2.2.1 Determining copolymerisation reaction time

The use of the Chemspeed Swing-SLT automated parallel synthesizer at CSIRO allowed up to 16 reactions to be carried out at once. It was also possible to perform kinetic studies on these reactions to track reaction progress with time.



Scheme 3. RAFT polymerisation of BMA and MAA using the RAFT agent 4-cyano-4[(dodecylsulfanylthio carbonyl)sulfanyl] pentanoic acid and the initiator 4,4'-azobis(4-cyanovaleric acid) (ACVA) to give a BMA-co-MAA as a macroRAFT agent. a = approx. 4 (9: 1 BMA: MAA), 8 (4: 1 BMA: MAA) or 13 (2: 1 BMA: MAA), b = approx. 27 (2: 1 BMA: MAA), 31 (4: 1 BMA: MAA) or 33 (9: 1 BMA: MAA).

A kinetic study was performed to determine optimum reaction time. The study consisted of three reactions in which the target molecular weight of the copolymers was kept constant (5,000 Da) but the ratio of BMA: MAA was altered, 9: 1, 4: 1 and 2: 1. The kinetics of the polymerisation reaction were expected to be affected by the ratio of monomers used. The overall reaction time was 22 h with aliquots were taken at 10 h, 14 h and 18 h. Results are displayed in **Table 4**.

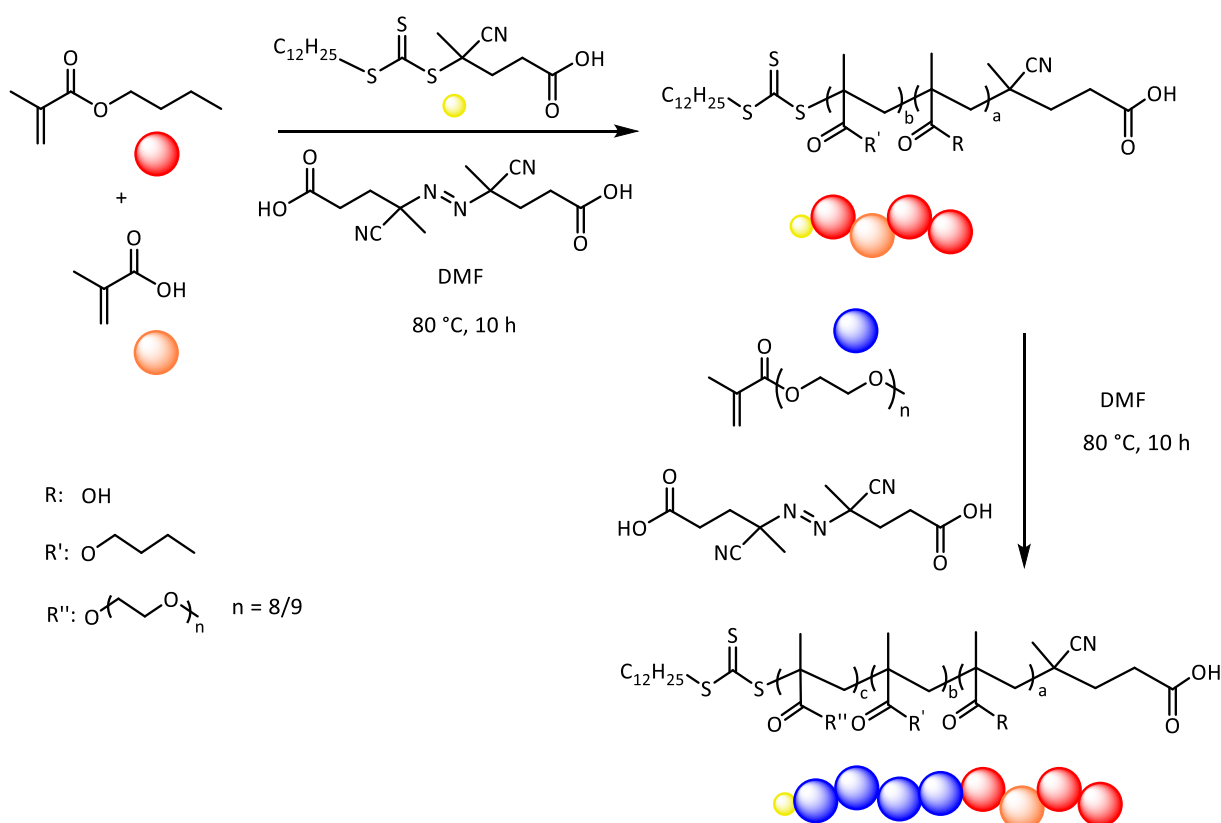
Monomer ratio (BMA: MAA)	Reaction time (h)	% conversion MAA	% conversion BMA	M_n (Da)	$\mathcal{D}_M$
9: 1	10	93	96	5,500	1.11
	14	93	96	5,600	1.11
	18	93	96	5,600	1.11
	22	93	96	5,700	1.11
4: 1	10	90	96	5,700	1.12
	14	93	97	5,800	1.12
	18	93	97	5,800	1.12
	22	93	98	5,900	1.12
2: 1	10	87	93	5,700	1.12
	14	89	94	5,800	1.13
	18	89	95	5,800	1.12
	22	89	96	5,900	1.13

Table 4. Analysis of copolymers synthesised in kinetic study. Percentage conversion was calculated from ^1H NMR spectra. M_n and $\mathcal{D}_M$ were determined via GPC (DMAc SEC).

Good levels of conversion were achieved (> 92%) and low dispersity polymers were synthesised (< 1.14). As percentage conversion values were high at the 10 h time point (> 90%), it was decided that future copolymerisation reactions would not be run for longer than 10 h. M_n values were higher than the target molecular weight of 5,000 Da. This discrepancy could be due to the fact that GPC M_n values are not absolute as they are calculated using a standard which is different in structure to the measured polymer. However, as the discrepancy is > 10% other causes should be considered. It is possible that human error, either in the preparation of stock solutions or in the programming of the Chemspeed, could have caused less RAFT agent to be added than the amount required to achieve 5,000 Da polymers. If less than the required amount of RAFT agent was added, then polymers with higher molecular weights than anticipated would be formed as it is the ratio of RAFT agent to monomer which controls the target molecular weight of the polymer.

2.2.2 Synthesis of quasi-block copolymers

The size of micelles formed from quasi-block copolymers will be compared to the size of micelles formed from pure-block copolymers to determine consistency in micelle formation.



Scheme 4. Synthesis of BMA-co-MAA-*b*-PEGMA in a two-step synthesis of a library of quasi-block copolymers. *a* = approx. 4 (9: 1 BMA: MAA), 8 (4: 1 BMA: MAA) or 13 (2: 1 BMA: MAA), *b* = approx. 33 (9: 1 BMA: MAA), 31 (4: 1 BMA: MAA) or 27 (2: 1 BMA: MAA), *c* = approx. 11 (5,000 Da), 21 (10,000 Da) or 33 (15,000 Da).

A one-pot synthesis of quasi-block copolymers was carried out to decrease the overall synthesis time. In this reaction, PEGMA was added to the BMA-co-MAA copolymer mixture after 10 h.

Three copolymers with different monomer ratios, 9: 1, 4: 1 and 2: 1, were synthesised in step one of the quasi-block copolymer synthesis. The target molecular weight of the copolymer block was kept constant (5,000 Da). The three copolymer macroRAFT agents synthesised in step one were used to synthesise nine quasi-block copolymers with varying PEGMA block molecular weights, details can be found in **Table 6** and a reactor schematic is shown in **Figure 21**.

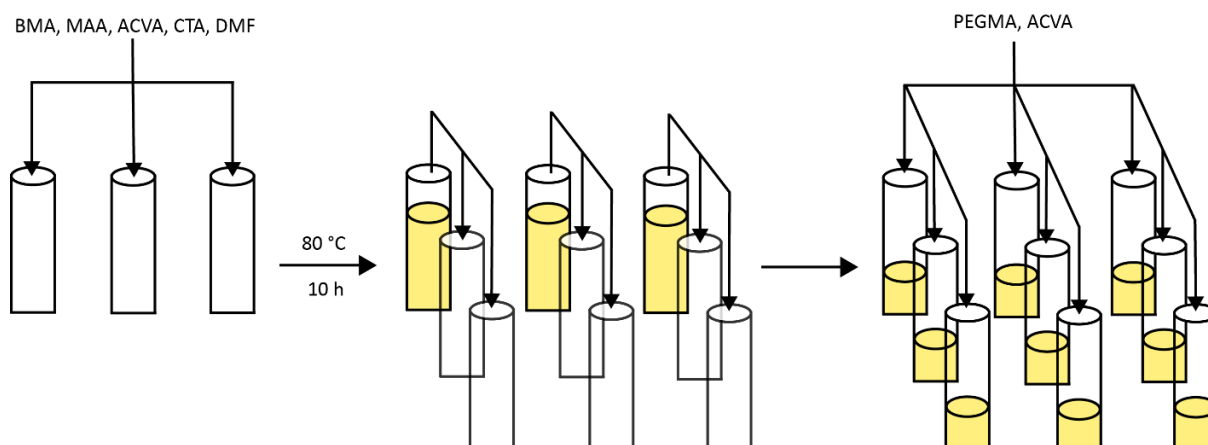


Figure 21. Representation of synthesis of quasi-block copolymer library via sequential RAFT polymerisation.

Copolymerisation results are displayed in **Table 5**, all copolymerisation reactions reached a good level of conversion (MMA: 91–92%, BMA: 96%) and had a low dispersity ($\mathcal{D}_M$) of approx. 1.13.

Reactor	Monomer ratio (BMA: MAA)	% conversion MAA	% conversion BMA	M_n (Da)	$\mathcal{D}_M$
1	9: 1	92	96	5,500	1.12
2	4: 1	91	96	5,700	1.12
3	2: 1	92	96	5,700	1.14

Table 5. Analysis of copolymers synthesised in step 1 of quasi-block copolymerisation reaction. Percentage conversion was calculated from ^1H NMR spectra. M_n and $\mathcal{D}_M$ determined using GPC analysis.

Following addition of the PEGMA monomer, GPC analysis of the quasi-block copolymers formed produced chromatograms in which the polymer peaks had shoulders (see **Figure 22**). In some cases, the shoulders were so significant that two separate peaks were detected (reactors 3, 7, 8 and 9) and polydispersity was > 1.20 for many samples. The shoulders seen in GPC traces were unreacted BMA-co-MAA copolymer which lost macroRAFT agent functionality, the RAFT agent end group was removed from the polymer chain (dead polymer chains). The elution times of the shoulders corresponded to the elution times of the copolymers, indicating that some of the copolymer sample was not chain extended in the second polymerisation reaction. Such dead polymer chains are a result of the use of initiator in the reaction.¹⁸⁵ Propagating radicals are formed from initiator and from the radical leaving group, R' , on the RAFT agent. Dead polymer chains are formed when there are more propagating radicals than there are RAFT agent end groups. For every molecule of RAFT agent used, one propagating radical is created (R'), therefore any propagating radicals formed from

the initiator result in more propagating radicals than RAFT end groups. Reducing the amount of initiator in the reaction reduces dead chain formation but will also reduce the rate of reaction.

It was noted that higher MAA concentrations resulted in larger amounts of unreacted BMA-co-MAA (larger shoulders can be seen in the GPC traces, **Figure 22**). It was hypothesised that the rate of fragmentation of propagating radicals from the RAFT agent was slower when higher levels of MAA were present in the reaction. This resulted in the formation of dormant polymer chains which were not able to fragment from the RAFT agent and therefore not able to chain extend.

Reactor	Monomer ratio (BMA: MAA)	Target MW of PEGMA block (Da)	Target MW of block copolymer (Da)	M_n (Da)	$\mathcal{D}_M$
1	9:1	5,000	10,000	9,000	1.16
2	4:1	5,000	10,000	11,400	1.26
3	2:1	5,000	10,000	11,300	1.11
				6,100	1.09
4	9:1	10,000	15,000	17,100	1.31
5	4:1	10,000	15,000	16,500	1.32
6	2:1	10,000	15,000	18,800	1.43
7	9:1	15,000	20,000	18,600	1.21
				6,600	1.07
8	4:1	15,000	20,000	25,400	1.25
				6,000	1.10
9	2:1	15,000	20,000	23,300	1.25
				6,100	1.09

Table 6. GPC analysis of quasi-block copolymers. M_n and $\mathcal{D}_M$ values calculated using GPC (DMAc SEC, LabSolutions software).

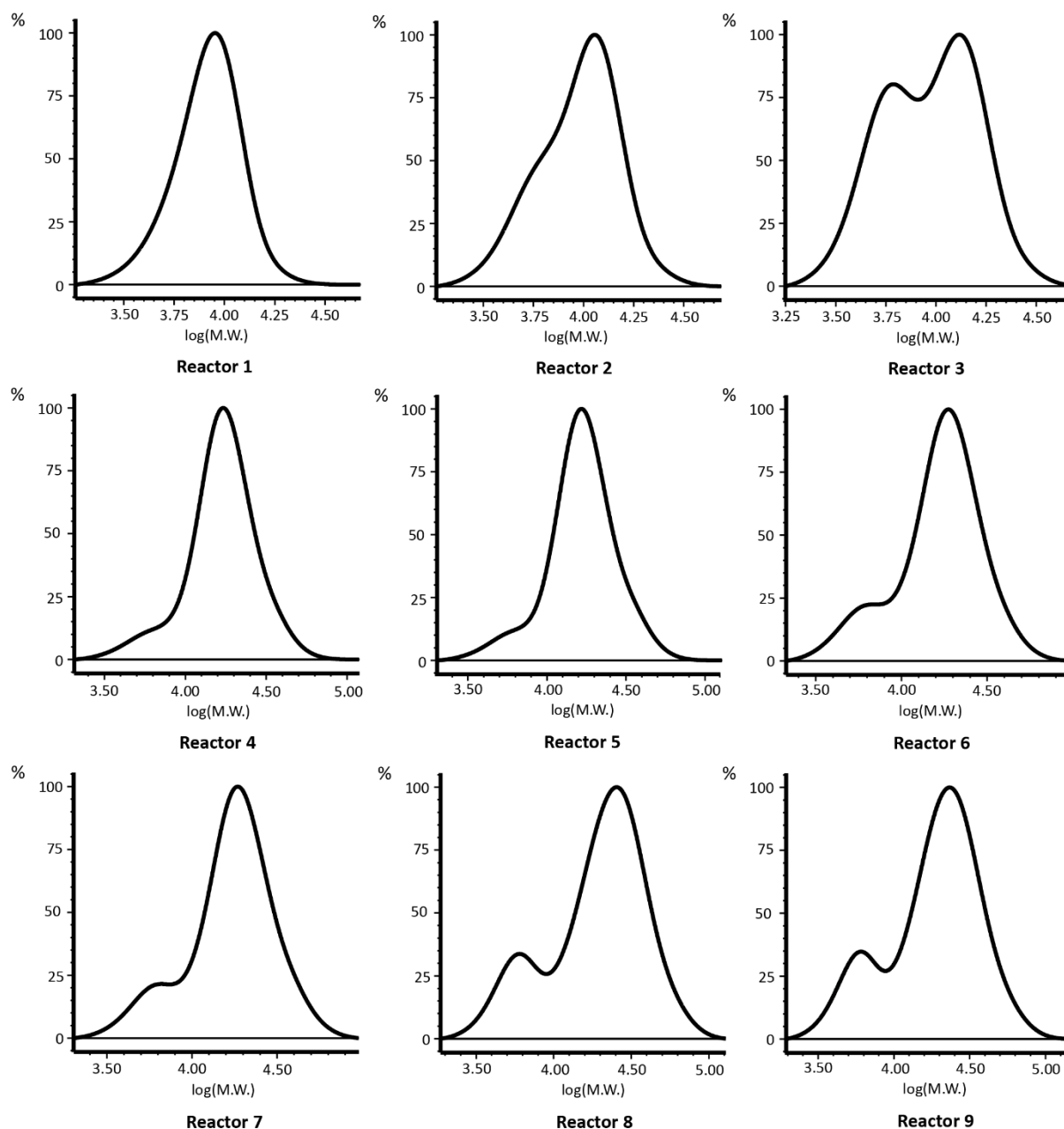


Figure 22. GPC (DMAc SEC) traces of synthesised quasi-block copolymers containing dead polymer chains.

2.2.3 Optimising reaction conditions

It was necessary to optimise reaction conditions to minimise dead chain formation. As high levels of propagating radicals and reaction time are both common causes of dead chain formation, both of these factors were investigated.¹⁸⁵ The number of propagating radicals in a reaction is largely determined by the type of, and concentration of, initiator used. The initiator type and concentration, and the length of the reaction were investigated in order to determine the optimum reaction conditions for the formation of BMA-co-MAA-*b*-PEGMA.

The copolymerisation step was the main focus of this study (rather than addition of the PEGMA monomer) as it was in this step that dead chain formation occurred.

Effect of initiator azobis(4-cyanovaleric acid) concentration on rate of reaction

Six reactions were studied in which the ratio of monomers and the concentration of azobis(4-cyanovaleric acid) (ACVA), in relation to the concentration of the RAFT agent, were varied to determine if high levels of conversion could still be reached with lower initiator concentrations. Initiator concentration is correlated with the formation of dead polymer chains.^{104,112} Reactions were run at 85 °C, the reaction time was 8 h and aliquots were taken at 1 h, 2 h, 4 h and 6 h. Different BMA: MAA ratios (9: 1, 4: 1 and 2: 1) were used as this was previously determined to have an effect on the rate of reaction; higher ratios of MAA resulted in faster reaction rates. The target molecular weight of the copolymers was kept constant (5,000 Da). Reagent ratios and results are shown in **Table 7**.

Reactor	Monomer ratio (BMA: MAA)	RAFT: initiator ratio	Reaction time (h)	% conversion BMA	M_n (Da)	$\bar{D}_M$
1	9: 1	1: 0.1	1	35	3,100	1.09
			2	52	3,700	1.10
			4	73	4,300	1.11
			6	77	4,600	1.12
			8	80	4,700	1.12
2	4: 1	1: 0.1	1	40	3,500	1.10
			2	58	4,200	1.12
			4	75	4,800	1.12
			6	79	5,100	1.13
			8	81	5,200	1.13
3	2: 1	1: 0.1	1	38	3,700	1.10
			2	57	4,300	1.12
			4	73	5,000	1.12
			6	77	5,200	1.12
			8	79	5,200	1.14
4	9: 1	2: 0.1	1	26	2,800	1.07
			2	37	3,200	1.09
			4	55	3,600	1.11
			6	59	3,800	1.11
			8	60	4,000	1.12
5	4: 1	2: 0.1	1	24	3,000	1.07
			2	28	3,100	1.09
			4	40	3,400	1.11
			6	42	3,500	1.11
			8	43	3,600	1.11
6	2: 1	2: 0.1	1	28	3,300	1.09
			2	32	3,400	1.09
			4	41	3,700	1.11
			6	43	3,700	1.11
			8	44	3,800	1.12

Table 7. Monomer and initiator ratios and analysis of copolymers. Percentage conversion was calculated from ^1H NMR spectra and M_n and $\bar{D}_M$ from GPC (DMAc SEC, LabSolutions software). It was not possible to calculate MAA % conversion in this study as the trioxane internal standard peak could not be isolated due to overlap of the peak with an impurity in the 0 h NMR spectrum. As described in **2.4.1**, it is possible to calculate BMA % conversion without an internal standard but not MAA % conversion.

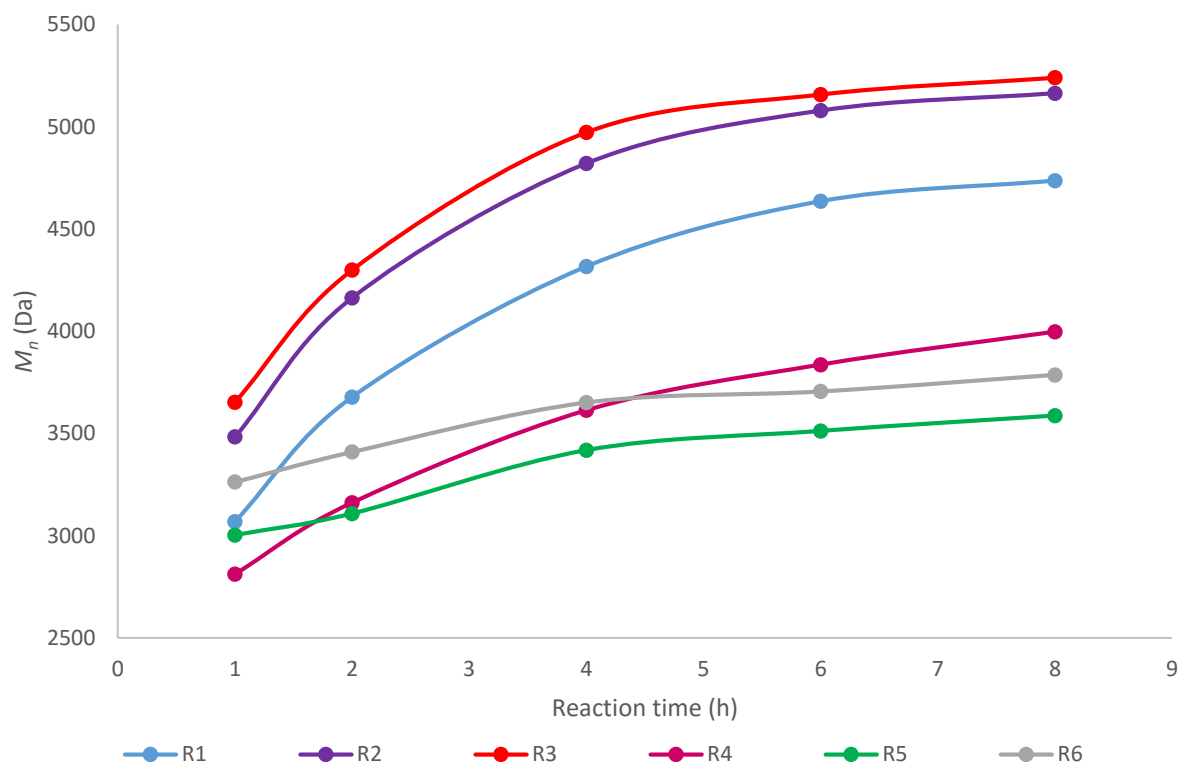
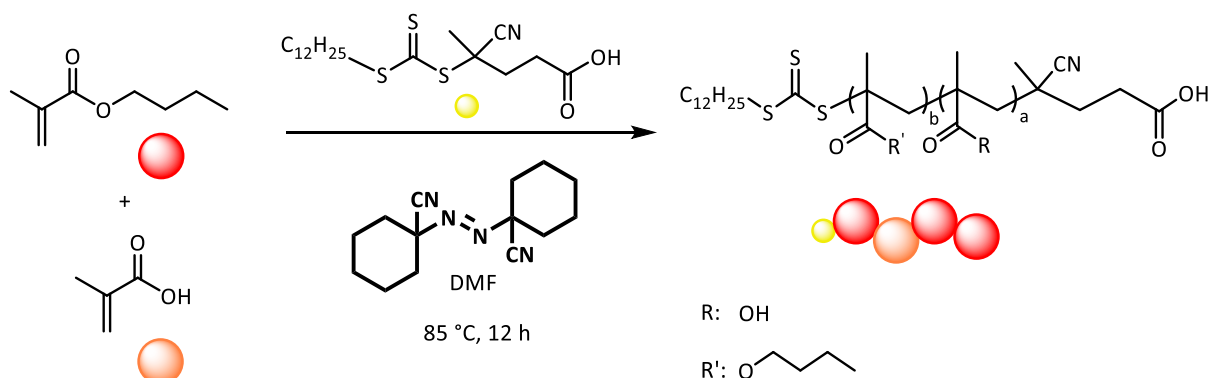


Figure 23. M_n compared to reaction time for 6 copolymerisations with varying initiator concentrations and monomer ratios. R1 had 10: 1 RAFT: initiator and 9: 1 BMA: MAA ratios. R2 had 1.0: 0.1 RAFT: initiator and 4: 1 BMA: MAA ratios. R3 had 10: 1 RAFT: initiator and 2: 1 BMA: MAA ratios. R4 had 20: 1 RAFT: initiator and 9: 1 BMA: MAA ratios. R5 had 2.0: 0.1 RAFT: initiator and 4: 1 BMA: MAA ratios. R6 had 20: 1 RAFT: initiator and 2: 1 BMA: MAA ratios.

A plot of reaction time versus polymer molecular weight is displayed in **Figure 23**. GPC analysis indicated that a molecular weight of approx. 5,000 Da to have been reached after 6 h, in the reactions containing initiator in a 1.0: 0.1 (RAFT: initiator) ratio and BMA: MAA ratios of 4: 1 and 2: 1. All polymers had a low polydispersity, < 1.15. In the reactors containing initiator in a 2.0: 0.1 (RAFT: initiator) ratio the molecular weight of the copolymer was approximately 4,000 Da after 8 h. When low initiator concentrations were used reaction times greater than 8 h were required to reach the target molecular weight of the copolymer. As reducing initiator concentration greatly reduced the rate of reaction it was determined that this was not an ideal option to reduce dead chain formation. R 1, 2 and 3 (9: 1, 4: 1 and 2: 1 respectively) had similar rates of reaction, although the conversion of monomers in R1 was lower throughout the reaction.

Azobis(cyclohexanecarbonitrile) as the initiator



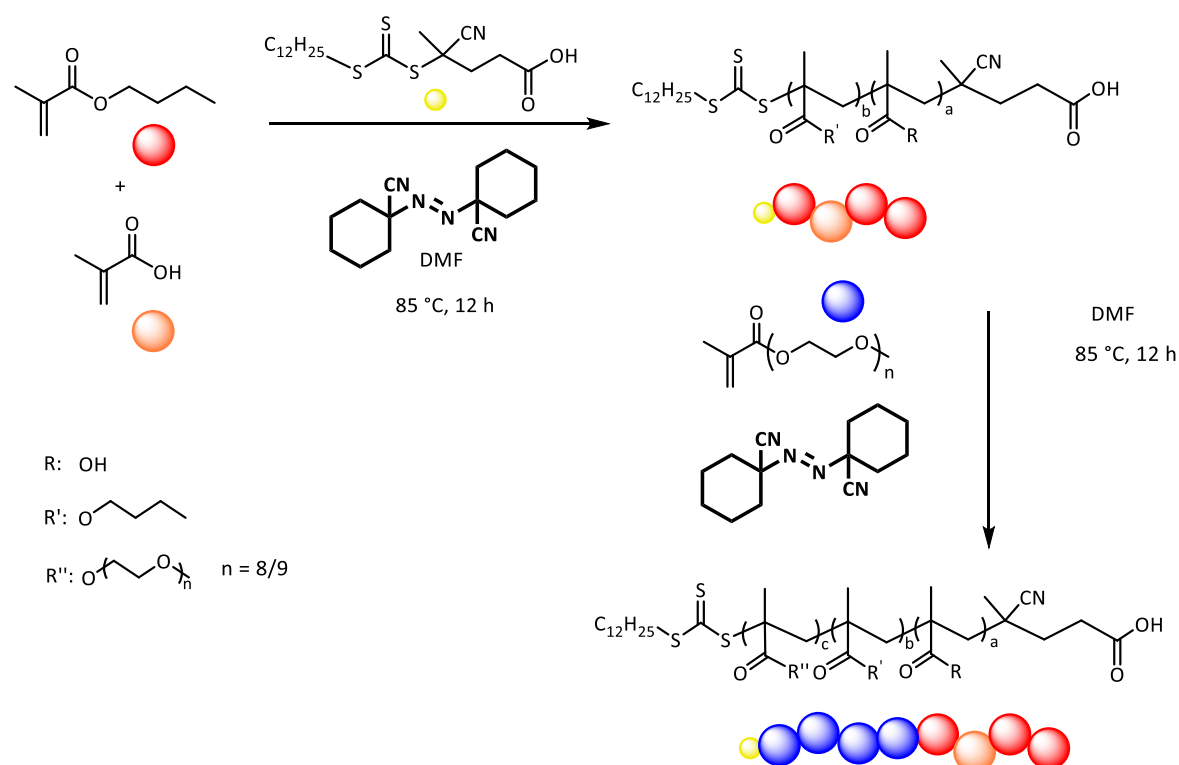
Scheme 5. Copolymerisation of BMA and MAA using the initiator ACHN (azobis(cyclohexanecarbonitrile)). a = approx. 4 (9: 1 BMA: MAA), 8 (4: 1 BMA: MAA) or 13 (2: 1 BMA: MAA), b = approx. 33 (9: 1 BMA: MAA), 31 (4: 1 BMA: MAA) or 27 (2: 1 BMA: MAA).

As reducing initiator concentration did not provide a constructive solution to dead chain formation during copolymerisation, the initiator azobis(cyclohexanecarbonitrile) (ACHN) was investigated. ACHN has a 10 h half-life at 88 °C as opposed to ACVA's 10 h half-life at 67 °C. The use of ACHN should lead to less dead chain formation as the rate of radical initiation will be slower. A slower rate of radical initiation results in less propagating radicals in the reaction at any one time; less propagating radicals means less radical–radical terminations and therefore less dead polymer chains. This experiment was performed to determine if BMA-co-MAA copolymerisation reactions using ACHN could reach conversion levels similar to those reached when ACVA was used. Three reactions were carried out, the reaction time was 12 h, a longer reaction time was used than when ACVA was the initiator as radical propagation, and therefore the reaction rate, was expected to be slower when ACHN was used. Monomer composition was varied (9: 1, 4: 1 and 2: 1 BMA: MAA). The target molecular weight of the copolymers was kept constant (5,000 Da). Results are displayed in **Table 8**; a good level of conversion and low dispersity was achieved (80–90%, ≤ 1.2). As similar levels of conversion were reached when ACHN and ACVA were used, ACHN was determined to be a suitable substitute for ACVA in the first polymerisation reaction. ACHN was therefore used in further reactions.

Reactor	Monomer ratio (BMA: MAA)	% conversion BMA	M_n (Da)	$\bar{D}_M$
1	9: 1	80	5,300	1.13
2	4: 1	87	6,800	1.18
3	2: 1	88	6,900	1.20

Table 8. Percentage conversion and GPC results for trials using initiator ACHN, reaction was run for 12 h. Percentage conversion values were calculated using ^1H NMR spectra. M_n and $\bar{D}_M$ values calculated using GPC (DMAc SEC, LabSolutions software).

Synthesis of quasi-block copolymers using ACHN



Scheme 6. Two step synthesis of BMA-co-MAA-*b*-PEGMA. ACHN used as the initiator. *a* = approx. 4 (5,000 Da), 5 (7,500 Da), 7 (10,000 Da) or 11 (15,000 Da). *b* = approx. 33 (5,000 Da), 49 (7,500 Da), 66 (10,000 Da) or 99 (15,000 Da). *c* = approx. 11 (5,000 Da), 21 (10,000 Da), 16 (7,500 Da) or 33 (15,000 Da).

A library of seven quasi-block copolymers was synthesised using the initiator ACHN in place of ACVA. The BMA: MAA monomer ratio was kept constant and the target molecular weight of BMA-co-MAA and PEGMA blocks were varied. The BMA: MAA ratio used was 9: 1, this was chosen as higher ratios of MAA previously led to the formation of a higher proportion of dead chains. The target molecular weight of the polymer blocks was varied to determine the effect of polymer molecular weight on micelle size. Seven copolymers were synthesised in step one of the reaction (**Scheme 6**), aliquots of the reaction mixture were taken before PEGMA and a second portion of ACHN were added to the reactors and the second block was synthesised.

Results are displayed in **Table 9**, a library of quasi-block copolymers was successfully synthesised but small shoulders, indicating dead chain formation, were seen in the GPC of block copolymers (**Figure 24**). Dead chain formation was reduced by the use of ACHN instead of ACVA but not eliminated. Further studies were required to optimise the reaction conditions.

Reactor	Target MW BMA-co-MAA block (Da)	BMA-co-MAA M_n (Da)	Target MW of PEGMA block (Da)	PEGMA block M_n (Da)	Target MW of block copolymer (Da)	Block copolymer M_n (Da)	$\mathcal{D}_M$
1	5,000	5,600	5,000	7,800	10,000	12,800	1.36
2	5,000	5,400	10,000	15,400	15,000	20,800	1.44
3	5,000	5,600	15,000	17,300	20,000	22,900	1.62
4	7,500	8,100	7,500	8,800	15,000	16,900	1.31
5	10,000	10,300	5,000	8,400	15,000	18,700	1.30
6	10,000	10,200	10,000	29,300	20,000	39,500	1.10
7	15,000	14,400	5,000	9,100	20,000	23,500	1.28

Table 9. GPC analysis of quasi-block copolymers. M_n and $\mathcal{D}_M$ values calculated using GPC (DMAc SEC, LabSolutions software).

In some cases, M_n values were higher than target molecular weights, GPC M_n values are approximate as they are calculated using standards with different in structure to the measured polymer. GPC analysis is an important tool for comparison between synthesised polymers with the same composition but does not provide absolute molecular weights. However, as discussed previously, discrepancies > 10% are possibly due to human or Chemspeed error. Polydispersity values were observed to be higher for block copolymers BMA-co-MAA-*b*-PEGMA than for copolymers BMA-co-MAA. This is due to the fact that the PEGMA ‘monomer’ is actually a polymer and therefore already has an associated polydispersity value which affects the polydispersity of the synthesised block copolymer.

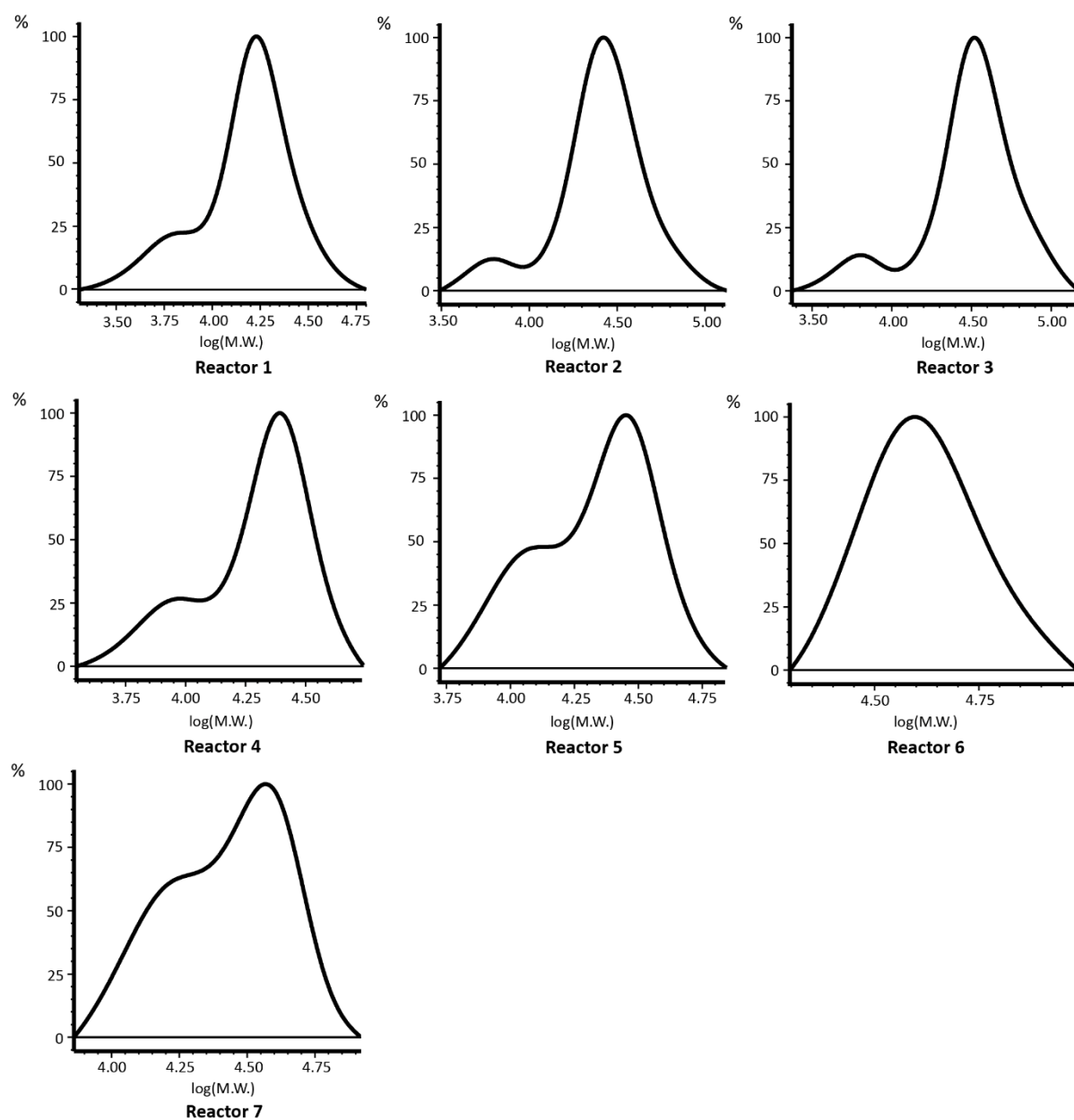
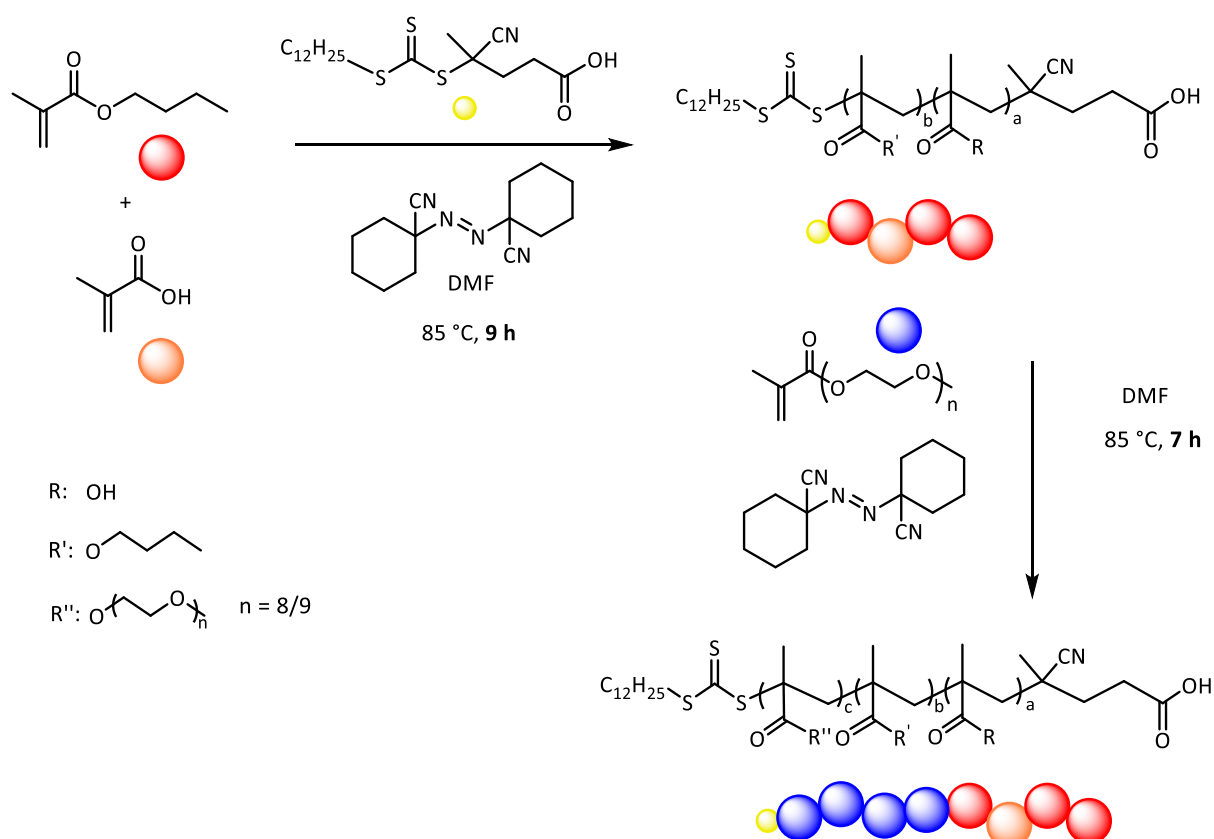


Figure 24. GPC chromatograms of quasi-block copolymers synthesised using ACHN. Proportion of dead chains, seen as shoulders on the peaks, reduced compared to when ACVA used (**Figure 22**).

Optimising reaction time and ACHN concentration



Scheme 7. Two step synthesis of BMA-co-MAA-*b*-PEGMA. In step one reaction time was reduced from 12 h to 9 h and seven out of 14 reactors had reduced ACHN concentrations of 1: 0.075 RAFT: initiator. a = approx. 4 (5,000 Da), 5 (7,500 Da), 7 (10,000 Da) or 11 (15,000 Da). b = approx. 33 (5,000 Da), 49 (7,500 Da), 66 (10,000 Da) or 99 (15,000 Da). c = approx. 11 (5,000 Da), 21 (10,000 Da), 16 (7,500 Da) or 33 (15,000 Da).

The use of the initiator ACHN instead of ACVA reduced the amount of dead chain formation occurring in copolymerisation reactions but did not solve the problem completely. As dead polymer chain formation can normally be attributed to either too much initiator or the reaction time being too long, a study into the effects of these two factors on dead chain formation was carried out. Each set of reactions had a range of target MWs (**Table 10**). The ratio of monomers in the copolymer block was kept constant, 9: 1 BMA: MAA (higher ratios of MAA correlated with the formation of more dead chains). One set of reactions were run (1–7) with reduced reaction time, 9 h, and a 1: 0.1 RAFT: initiator ratio and one set of reactions were run (8–14) with reduced reaction time, 9 h, and reduced initiator concentration, RAFT: initiator 1: 0.075 instead of 1: 0.10. The formation of dead polymer chains was only ever an issue in the first step (BMA-co-MAA synthesis) so the initiator

concentration in the second step (addition of PEGMA block) was not altered. The block copolymerisation was left for 7 h, opposed to 12 h as previous work carried out in the RAFT group at CSIRO suggested that a high percentage of the PEGMA would be converted after 7 h so there was no need to run the reaction for longer.

Reactor	Target MW BMA-co-MAA block (Da)	BMA-co-MAA M_n (Da)	Target MW PEGMA block (Da)	PEGMA block M_n (Da)	Target MW of block copolymer (Da)	Block copolymer M_n (Da)	$\mathcal{D}_M$
1	5,000	4,900	5,000	5,500	10,000	10,400	1.26
2	5,000	4,900	10,000	7,100	15,000	12,000	1.33
3	5,000	4,900	15,000	9,300	20,000	14,200	1.39
4	7,500	6,600	7,500	6,000	15,000	12,600	1.27
5	10,000	8,200	5,000	5,900	15,000	14,100	1.28
6	10,000	7,400	10,000	8,800	20,000	16,200	1.29
7	15,000	9,100	5,000	6,900	20,000	16,000	1.27
8	5,000	5,000	5,000	5,400	10,000	10,400	1.32
9	5,000	5,100	10,000	7,600	15,000	12,700	1.59
10	5,000	4,600	15,000	10,800	20,000	15,400	1.36
11	7,500	5,900	7,500	6,800	15,000	12,700	1.27
12	10,000	7,900	5,000	6,200	15,000	14,100	1.30
13	10,000	7,600	10,000	7,800	20,000	15,400	1.33
14	15,000	9,500	5,000	5,100	20,000	14,600	1.29

Table 10. Target molecular weights (MW) and GPC results for synthesised quasi-block copolymers. Samples displayed in blue were selected for micellar self-assembly studies. M_n and $\mathcal{D}_M$ values calculated using GPC (DMAc SEC, LabSolutions software).

There were no significant shoulders seen in block copolymer GPC traces for reactions 1–14, this indicated that dead chains were not formed in the copolymerisation reaction (**Figure 25**). GPC analysis showed that, while the higher target molecular weights were not reached, a set of polymers with a range of molecular weights was synthesised and, significantly, dead chains were not formed in the first block copolymer reaction. Further reactions were therefore run for 9 h. A collection of polymers with a range of molecular weights were selected to investigate if micelles could be assembled from quasi-block copolymers and to determine the effect of block molecular weight on micelle size. Shorter (9 h) reaction times, the use of the initiator ACHN in the place of ACVA and low MAA concentrations helped reduced the formation of dead polymer chains in the BMA-co-MAA polymerisation step. The formation of dead polymer chains was caused by radical–radical terminations which occurred when the concentration of propagating radicals was too high, this was a result of too many initiating radicals being formed as the initiator decomposed too quickly.

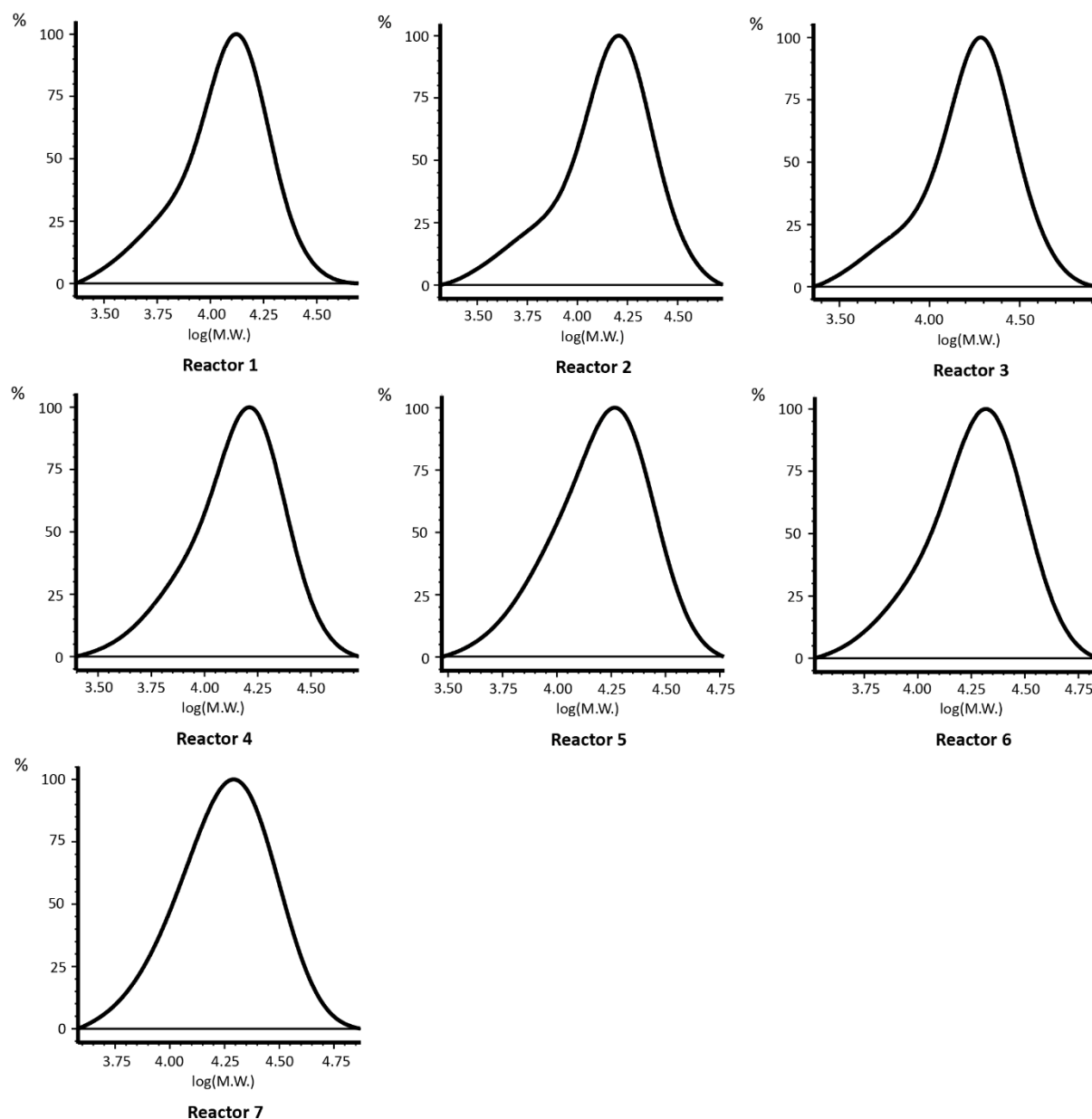


Figure 25. GPC traces from quasi-block copolymers synthesised using the initiator ACHN with a 9 h reaction time.

Bench synthesised polymers

Once the reaction conditions had been established a number of pure and quasi-block copolymers were synthesised on the bench at Imperial College London, to expand the polymer library. The polymer details are displayed in **Table 11**. Polymers with a range of monomer ratios and block lengths were synthesised using optimised reaction conditions (**Scheme 7**). All polymers displayed in **Table 11** were used in micelle self-assembly studies, section **2.2.4**. Polymers all had a low polydispersity (1.08–1.24).

Synthesis	BMA: MAA	BMA-co-MAA M_n (Da)	PEGMA block M_n (Da)	Block copolymer M_n (Da)	$\bar{D}_M$
PB	2: 1	3,100	700	3,800	1.18
PB	2: 1	3,900	1,500	5,400	1.17
PB	4: 1	4,200	1,700	5,900	1.19
PB	9: 1	5,300	1,100	6,400	1.12
QB	9: 1	2,100	4,400	6,500	1.24
PB	9: 1	4,300	2,400	6,700	1.08
QB	4: 1	4,500	2,800	7,300	1.12
QB	9: 1	5,300	2,700	8,000	1.12
PB	4: 1	6,500	2,400	8,900	1.22
QB	2: 1	4,000	5,500	9,500	1.14

Table 11. Bench synthesised polymers, QB is quasi-block and PB is pure-block. M_n and $\bar{D}_M$ values calculated using GPC (THF SEC, Waters Empower-3 software).

2.2.4 Self-assembly of micelles

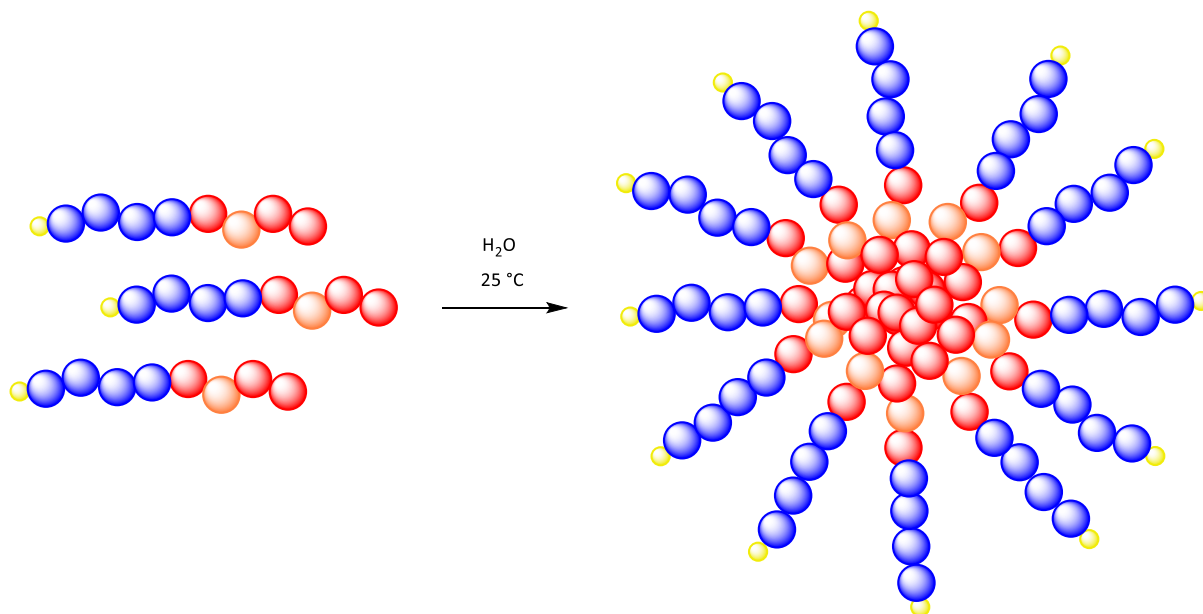


Figure 26. Self-assembly of micelles in water, from synthesised block copolymers.

Dynamic light scattering (DLS) analysis was used to assess the ability of the synthesised polymers to form micelles. Polymers were dissolved in water (0.5 mg/ml) and assembled micelles were measured. DLS showed that quasi-block copolymers self-assembled into micelle particles. A range of micelle sizes were formed (30–170 nm). A correlation was found between the polymer molecular weight and the size of the micelle particle formed, higher molecular weight polymers formed larger micelle particles (see **Figure 27**).

Pure-block copolymer micelles vs quasi-block copolymer micelles

Micelles formed from pure and quasi-block copolymers were compared to determine whether the method of synthesis had an effect on the micelle's structure and properties. Polymers were dissolved in water (0.5 mg/ml) and DLS was used to measure the micelles formed. Pure-block and quasi-block copolymers formed similar size micelles, with the exception of the 9,100 Da quasi-block copolymer which was much smaller than the 9,400 Da pure-block copolymer, see **Table 12**. DLS analysis of the 9,100 Da quasi-block and 9,400 Da pure-block copolymers was repeated but the different sized micelles were still formed, this result was believed to be an anomaly due to the close size correspondence of all other pure- and quasi-block micelles tested.

QB Total M_n (Da)	M_n of BMA-co-MAA block (Da)	M_n of PEGMA block (Da)	Micelle diameter (nm)	PB Total M_n (Da)	M_n of BMA-co-MAA block (Da)	M_n of PEGMA block (Da)	Micelle diameter (nm)
7,400	4,300	3,100	35 ±16	7,100	4,500	2,600	33 ±13
10,000	4,400	5,600	49 ±24	9,500	4,700	4,800	44 ±20
9,100	7,000	2,100	45 ±17	9,400	7,700	1,700	112 ±73
11,100	7,100	4,000	113 ±72	11,400	7,400	4,000	130 ±66

Table 12. Comparison between micelles formed from pure-block (PB) copolymers and quasi-block (QB) copolymers. In pure-block copolymer synthesis, copolymers were purified via dialysis and isolated via lyophilisation. M_n and $\bar{M}_w$ values calculated using GPC (THF SEC, Waters Empower-3 software). Micelle diameters were measured using DLS.

Effect of polymer molecular weight on micelle size

Analysis of micelles formed from quasi-block copolymers showed a general trend between the molecular weight of the polymer chain and the size of the micelle particle formed, higher molecular weight polymers led to larger micelles (**Figure 27**). The 170 nm micelle was identified as an outlier.

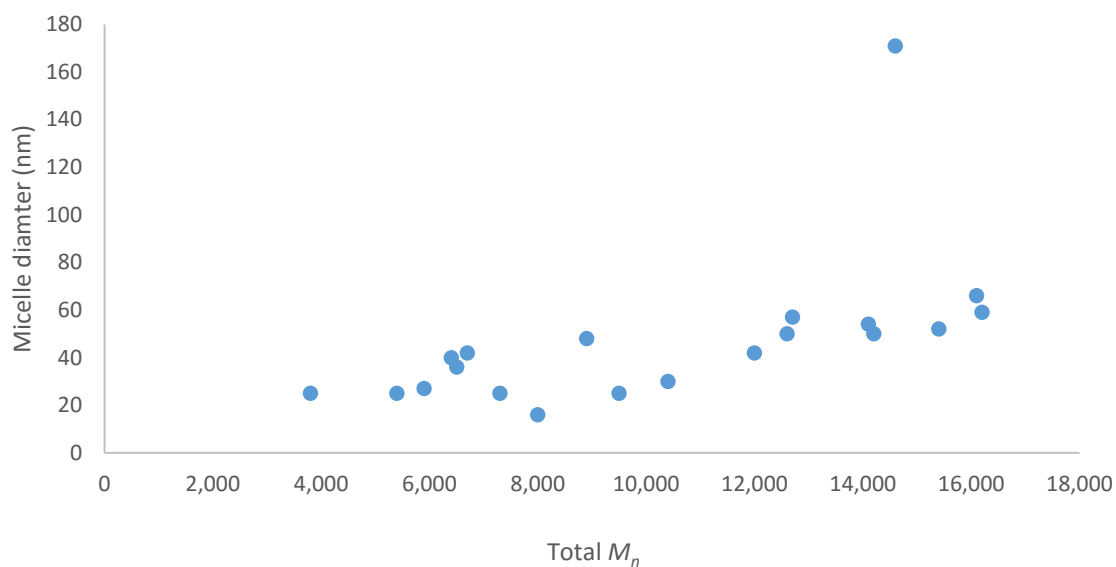


Figure 27. Polymer molecular weight versus micelle size. 170 nm micelle identified as outlier.

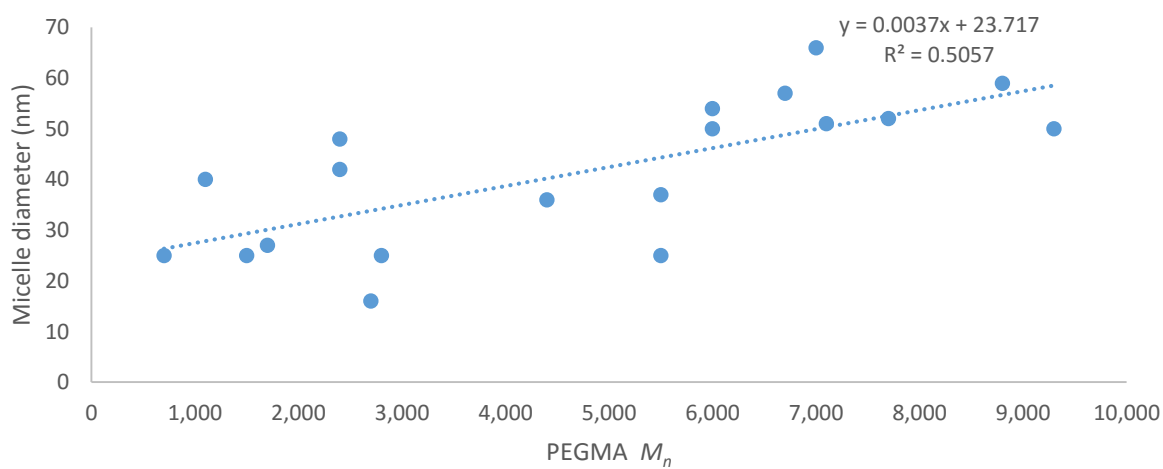
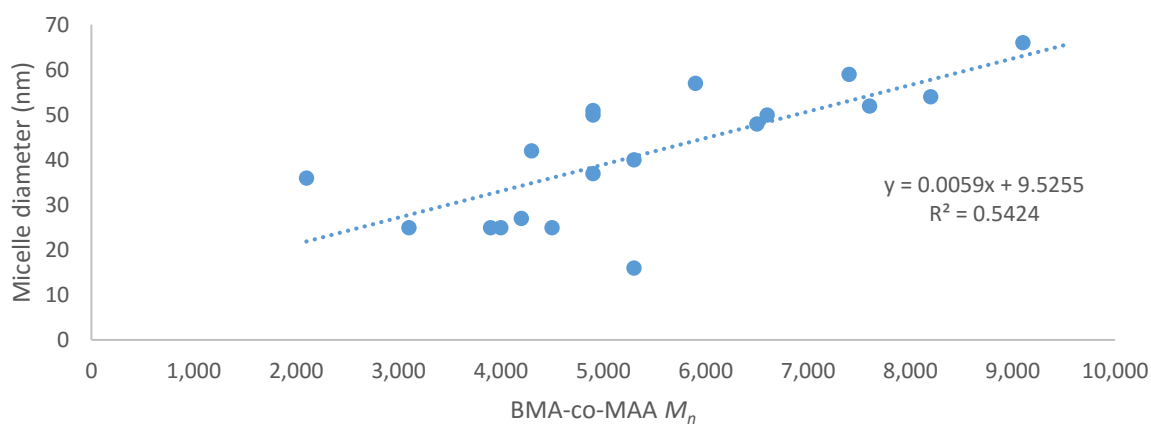
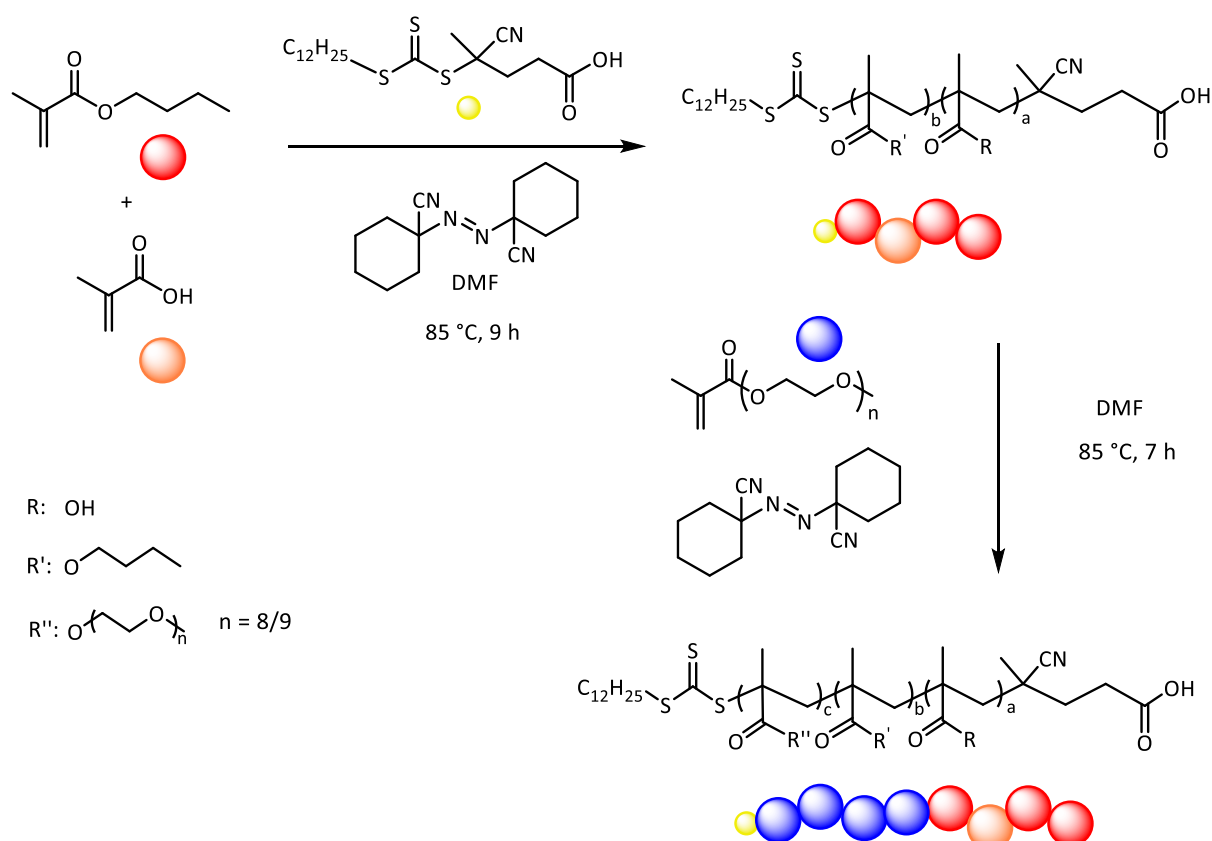


Figure 28. BMA-co-MAA copolymer block molecular weight versus micelle size (top) and PEGMA block molecular weight versus micelle size (bottom).

It was determined from results displayed in **Figure 28** that the hydrophobic BMA-co-MAA block had a more significant effect on micelle size than the hydrophilic PEGMA block. The gradient of the slope is steeper and R^2 is larger in the micelle diameter versus BMA-co-MAA molecular weight plot compared to the micelle diameter versus PEGMA molecular weight plot. This result corresponds with literature findings that the hydrophobic block has a more significant effect on micelle size than the hydrophobic block.^{177–181}

Synthesis of higher molecular weight polymers



Scheme 8. BMA-co-MAA-*b*-PEGMA copolymer synthesis. a = approx. 7 (10,000 Da, 9: 1 BMA: MAA), 15 (20,000 Da, 9: 1 BMA: MAA), 22 (30,000 Da, 9: 1 BMA: MAA), 27 (10,000 Da, 2: 1 BMA: MAA), 54 (20,000 Da, 2: 1 BMA: MAA) or 81 (30,000 Da, 2: 1 BMA: MAA). b = approx. 66 (10,000 Da, 9: 1 BMA: MAA), 132 (20,000 Da, 9: 1 BMA: MAA), 198 (30,000 Da, 9: 1 BMA: MAA), 54 (10,000 Da, 2: 1 BMA: MAA), 108 (20,000 Da, 2: 1 BMA: MAA) or 162 (30,000 Da, 2: 1 BMA: MAA) c = 11 (5,000 Da), 21 (10,000 Da) or 32 (15,000 Da).

The maximum micelle size observed was 170 nm. However, the literature suggests that for selective delivery to macrophages, larger (at least 500 nm) micelles are necessary. For this reason an attempt was made to synthesize larger micelles.^{63,96–98} In the largest particle

previously synthesised (diameter 170 nm), the copolymer block of the polymer had a molecular weight of 9,500 Da and the PEGMA block was 5,000 Da, making the overall molecular weight 14,500 Da. This ratio of approximately 2: 1, BMA-co-MAA block: PEGMA block, was used in the attempted synthesis of larger micelle particles. As preliminary work, carried out by Robert Davidson in the Fuchter group, determined that varying the BMA: MAA ratio in the copolymer block had an effect on micelle particle size, with a ratio of 2: 1 BMA: MAA forming the largest micelles, higher molecular weight polymers with this BMA: MAA ratio were also synthesised (see **Table 13**). Although larger molecular weight polymers were synthesised successfully, polymers with high target molecular weights had low percentage monomer conversion.

BMA: MAA ratio	Target MW (Da)	M_n (Da)	$\bar{D}_M$	Average micelle dia. (nm)
9: 1	15,000	11,900	1.13	115 ±59
9: 1	30,000	17,900	1.14	170 ±30
9: 1	45,000	25,900	1.13	122 ±50
2: 1	30,000	13,600	1.18	159 ±13
2: 1	45,000	24,800	1.12	119 ±53

Table 13. Quasi-block copolymers synthesised with higher molecular weights (MW) in an attempt to form micelle particles > 170 nm. M_n and $\bar{D}_M$ values calculated using GPC (THF SEC, Waters Empower-3 software). Micelle diameters were measured using DLS.

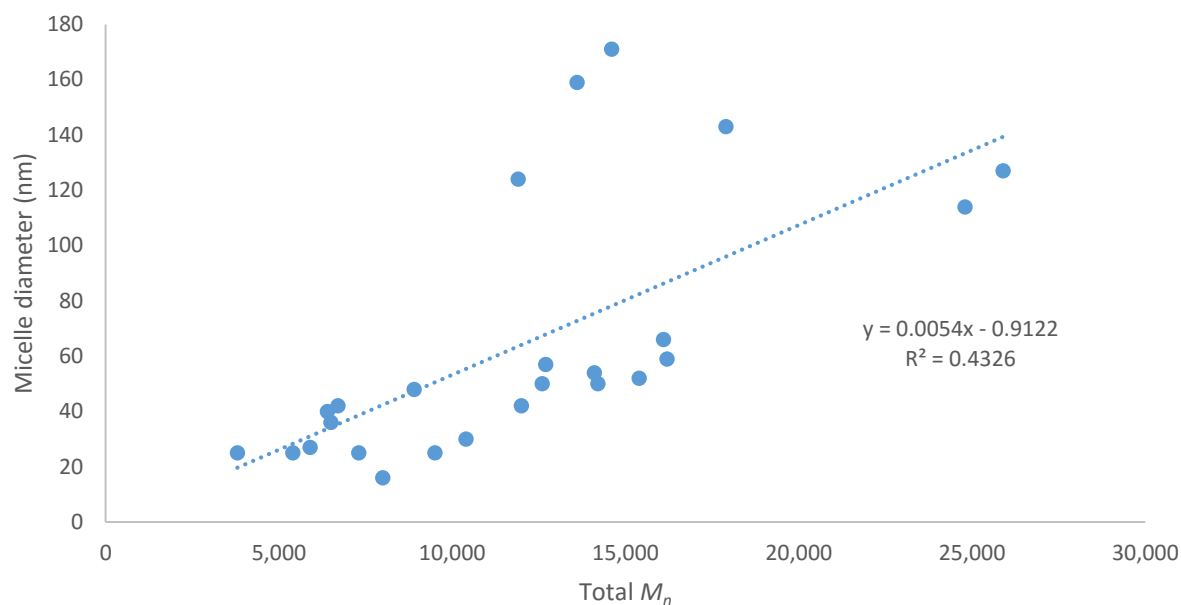
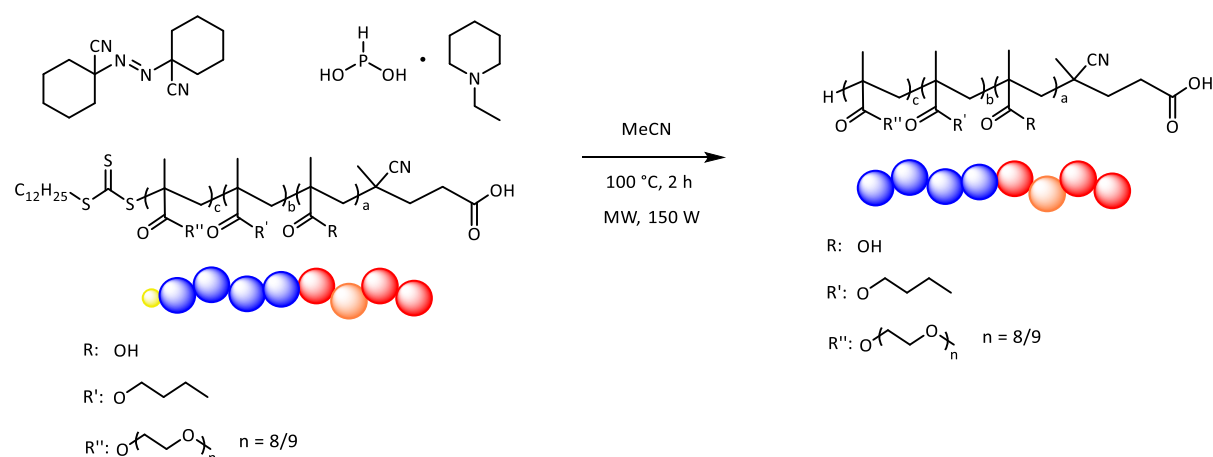


Figure 29. Polymer molecular weight (number averaged molecular weight) versus micelle size, including data for high molecular weight polymers.

Higher molecular weight polymer chains did not form micelle particles larger than 200 nm and a clear correlation between molecular weight and micelle diameter was not observed. Although the reason for this is unclear, it is possible that different length polymer chains pack differently in micelle self-assembly, for example larger chains may pack more closely than shorter chains.²⁰⁰

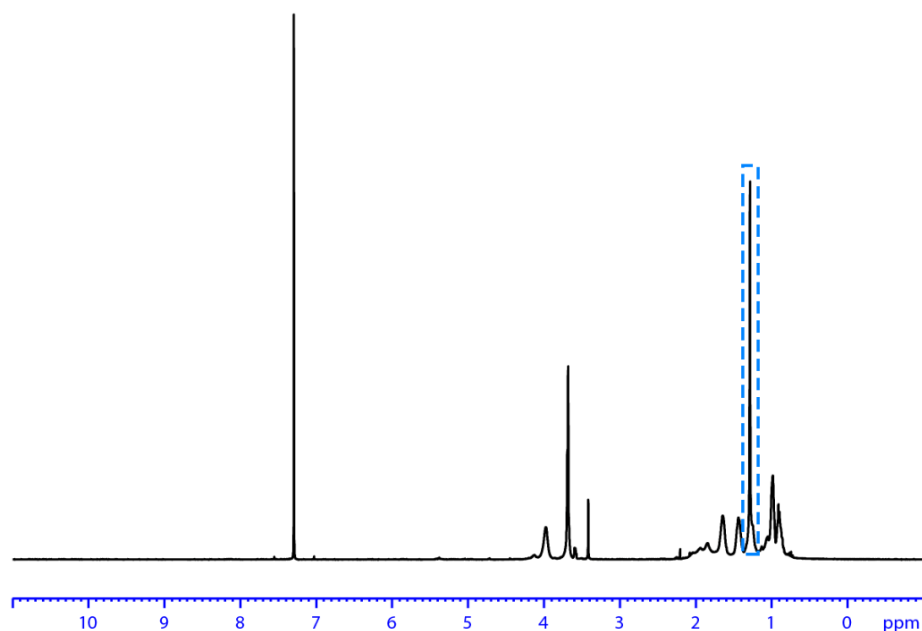
2.2.5 Removal of RAFT agent



Scheme 9. Removal of RAFT agent from polymer chains using ethyl piperidine hypophosphite and ACHN. Reaction was performed in the microwave. a = approx. 7 (10,000 Da, 9: 1 BMA: MAA), 15 (20,000 Da, 9: 1 BMA: MAA), 22 (30,000 Da, 9: 1 BMA: MAA), 27 (10,000 Da, 2: 1 BMA: MAA), or 81 (30,000 Da, 2: 1 BMA: MAA). b = approx. 66 (10,000 Da, 9: 1 BMA: MAA), 132 (20,000 Da, 9: 1 BMA: MAA), 198 (30,000 Da, 9: 1 BMA: MAA), 54 (10,000 Da, 2: 1 BMA: MAA), 108 (20,000 Da, 2: 1 BMA: MAA) or 162 (30,000 Da, 2: 1 BMA: MAA). c = 11 (5,000 Da) or 32 (15,000 Da).

The RAFT end group was removed from a number of quasi-block copolymers to analyse the effect that this would have on the size of the micelle formed. RAFT end group removal was achieved *via* reaction with initiator (ACHN), the initiator attacks the thiocarbonylthio group and replaces the polymer chain on the RAFT agent, the polymer chain then takes part in a radical-induced reduction with a hypophosphite salt.^{201–203} The disappearance of the peak from the hydrocarbon chain on the RAFT agent (δ 1.25) in the ^1H NMR indicated that the RAFT group was successfully removed, see **Figure 30** for example.

RAFT end group attached



RAFT end group removed

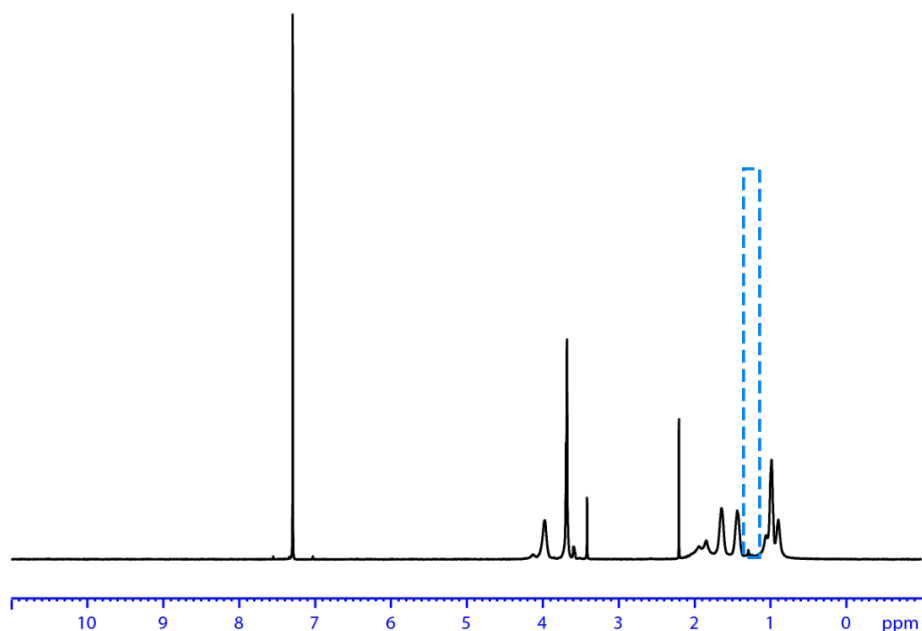


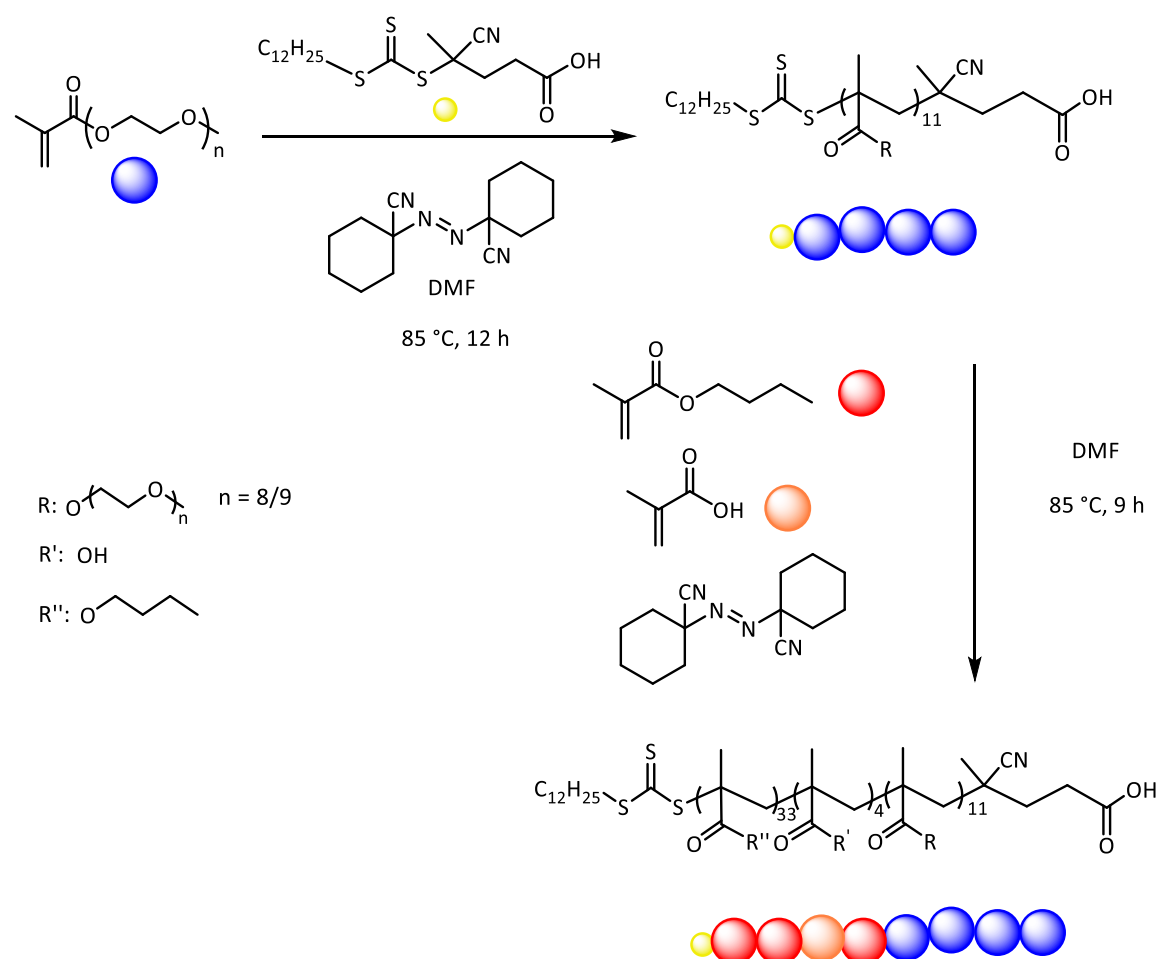
Figure 30. NMR spectra pre- and post-RAFT agent removal. Disappearance of RAFT agent hydrocarbon chain peak (δ 1.25) indicated that the RAFT agent had been removed. Spectrum of 11,900 Da polymer (9: 1 BMA: MAA) shown.

Micelle particles were generally larger once the RAFT end group was removed (**Table 14**). However, micelles were notably less soluble in water after the removal of the RAFT group, this could be responsible for their increase in size as it was determined in **Chapter III, 3.2.1** that micelles swell at low concentrations.

BMA: MAA ratio	M_n (Da)	$\bar{D}_M$	Micelle diameter RAFT end group attached (nm)	Micelle diameter RAFT end group removed (nm)
9: 1	11,900	1.13	124	139
9: 1	17,900	1.14	129	140
9: 1	25,900	1.13	123	140
2: 1	13,600	1.18	159	175
2: 1	24,800	1.12	92	133

Table 14. Micelle size before and after removal of RAFT group. M_n and $\bar{D}_M$ values calculated using GPC (THF SEC, Waters Empower-3 software). Micelle diameters were measured using DLS.

2.2.6 'Reverse' quasi-block copolymer synthesis



Scheme 10. Synthesis of PEGMA-*b*-BMA-co-MAA 'reverse' block copolymer via synthesis of PEGMA macroRAFT agent.

A 'reverse' quasi-block copolymer was successfully synthesised in which the copolymer block monomers were polymerised onto an already synthesised PEGMA block. This differs from the standard synthesis of quasi-block copolymers in this project as the BMA-co-MAA block was previously made first followed by a PEGMA chain extension. The aim of synthesising a reverse

quasi-block copolymer was to investigate whether they assembled into micelles that had similar diameters to micelles assembled from standard quasi-block copolymers. DLS analysis determined that the micelles formed from reverse quasi-block copolymers did have the same diameter as micelles formed from standard quasi-block copolymers, **Table 15**.

	Micelle diameter (nm)	M_n (Da)	$\bar{D}_M$
Standard quasi-block copolymer	37	7,500	1.16
Reverse quasi-block copolymer	36	7,400	1.22

Table 15. Comparison of standard and reverse quasi-block copolymers. Micelles with the similar diameters were formed. M_n and $\bar{D}_M$ values calculated using GPC (THF SEC, Waters Empower-3 software). Micelle diameters were measured using DLS.

2.3 Conclusions

Radical–radical terminations were seen at a high level in initial polymerisation reactions. Extensive kinetic studies determined that, by shortening reaction time and substituting the initiator ACVA for ACHN, this dead chain formation could be minimised. A library of quasi-block copolymers was synthesised using the optimised reaction conditions. A range of sizes of polymeric micelles were successfully self-assembled from quasi-block copolymers with varying polymer composition and chain length. Increased polymer molecular weight correlated with larger micelle particles at low polymer molecular weights but this trend was not consistent at higher molecular weights (> 16,500 Da). It was therefore not possible to synthesise micelle particles larger than approximately 200 nm. Micelles formed from ‘reverse’ quasi-block copolymers and quasi-block copolymers with the RAFT end-group removed were also studied in case the positioning of the RAFT agent at the end of the hydrophilic chain had an effect on the size of micelles formed, but this was not the case. Although micelle particles larger than 200 nm were not synthesised, a reasonable size range of micelles was obtained (15–170 nm). A library of different sized micelle particles was selected for cellular studies to determine the uptake of the micelles by macrophages. Prior to *in vitro* work, micelles had to be stabilised for use at high dilution and fluorescently tagged to allow *in vitro* tracking. The adaptation of micelles in preparation for cellular studies is outlined in **Chapter III** and biological studies and results are described in **Chapter IV**.

2.4 Experimental

2.4.1 Materials and methods

Reagents and solvents were supplied from commercial sources and used as delivered unless otherwise detailed. Commercial suppliers used were: Sigma Aldrich, Thermofisher Scientific and VWR.

All Chemspeed reactions were carried out using a Chemspeed Swing-SLT automated parallel synthesiser. The synthesiser was equipped with a glass reactor block consisting of 16 reaction vessels (13 ml) with thermal jackets connected in series through the reaction block and connected to a heating/cooling system (Hüber, -90 °C to 140 °C). In addition, all reaction vessels were equipped with cold-finger reflux condensers (approximately 7 °C). Mixing was achieved by vortex agitation (up to 1400 rpm). Liquid transfers were handled by a 4-needle head (4-NH) capable of four simultaneous sample transfers. The 4-NH was connected to a reservoir bottle (degassed DMF solvent) for needle rinsing after each liquid transfer step. This DMF solvent reservoir was degassed by continuous sparging with nitrogen and was also utilised to prime the tubing lines of the 4-NH. When experiments were carried out the synthesiser was maintained under an inert atmosphere by supplying a constant flow of nitrogen into the hood of the synthesiser. A nitrogen atmosphere was also applied to reactors and stock solutions at all times. Prior to the experiments, the reaction vessels were heated to 135 °C and subjected to 10 cycles of vacuum (2 min each) and filling with nitrogen (2 min each) to ensure the elimination of oxygen. After this pre-treatment, the RAFT polymerisation experiments were carried out.

The Chemspeed parallel synthesizer has an automated loading system so stock solutions, in DMF, of known concentrations were made up for experiments in which several reactions were carried out simultaneously. The Chemspeed was programmed to add the correct amount of each reactant to each reactor. In studies with three or less reactions the reaction mixtures were prepared and degassed on the bench and then added to the Chemspeed reactor.

Microwave reactions were performed in a CEM discover microwave synthesiser at 17.2 bar. The magnetron frequency was 2450 MHz. Reactions were agitated *via* electromagnetic

stirring with Teflon coated stir bars. 10 ml borosilicate glass reaction vessels were used with silicon septum caps (both purchased from CEM). Air cooling was used post reaction (≥ 25 psi, 20 l/min flow).

Proton nuclear magnetic resonance (^1H NMR) (400 MHz) spectra were recorded using a Bruker AV400 spectrometer at 25 °C in deuterated chloroform (CDCl_3) to determine monomer to polymer conversions and copolymer compositions. Trioxane was used as an internal standard.

The majority of gel permeation chromatography (GPC) detailed in this chapter was performed on a Shimadzu system equipped with a CMB-20A controller system, a SIL-20AHT autosampler, a LC-20AT tandem pump system, a DGU-20A degasser unit, a CTO-20AC column oven, a RDI-10A refractive index detector and 4 x Waters Styragel columns (HT2, HT3, HT4, HT5 each 300 mm x 7.8 mm, providing an effective molar mass range of $100\text{--}4 \times 10^6$). This SEC system used N,N-dimethylacetamide (DMAc) (with 2.1 g/l of lithium chloride (LiCl)) as elutant with a flow rate of 1ml/min at 80 °C. The molar mass of the samples was obtained from a calibration curve constructed with PMMA standards (Polymer Laboratories) of low dispersity ($\mathcal{D}$) value. A 3rd order polynomial was used to fit the $\log M_p$ vs. time calibration curve, which was linear across the molar mass ranges. LabSolutions software was used to process results and obtain M_n and $\mathcal{D}_M$ values.

A tetrahydrofuran (THF) GPC system was also used where specified: a Waters Alliance system, equipped with an Alliance 2695 Separations Module (integrated quaternary solvent delivery, solvent degasser and autosampler system), a Waters column heater module, a Waters 2414 RDI refractive index detector, a Waters PDA 2996 photodiode array detector (210 to 400 nm at 1.2 nm) and 4 x Agilent PL-Gel columns (3 x PL-Gel Mixed C (5 μm) and 1 x PL-Gel Mixed E (3 μm) columns), each 300 mm x 7.8 mm², providing an effective molar mass range of 200 to 2×10^6 . THF high purity solvent (HPLC grade) was pre-filtered through aluminium oxide (90 active neutral, 70—230 mesh) with a 0.45 μm filter, and 0.1 g/l 2,6-di-tert-butyl-4-methylphenol (BHT) was added as inhibitor. The filtered THF containing BHT was purged slowly with nitrogen gas and used as an eluent with a flow rate of 1 ml/min at 30 °C. Number average (M_n) and weight average (M_w) molar masses were evaluated using Waters Empower-3 software. The GPC columns were calibrated with low dispersity polystyrene (PSt)

standards (Polymer Laboratories) ranging from 580–7, 500,000 g/mol, and molar masses are reported as PSt equivalents. A 3rd-order polynomial was used to fit the $\log M_p$ vs. time calibration curve, which was near linear across the molar mass ranges.

DLS measurements were performed on a Beckman Coulter DelsaNano C Particle Analyzer. BRAND 1.5 ml semi-micro polystyrene cuvettes were used. All solutions were filtered through 0.45 μm Supor Membrane 25 mm syringe filters (Acrodisc). *Measurement parameters*: repetition: 5, temperature: 25 °C, equilibration: 20 sec, wait time: 120 sec, dust limit: 10, upper dust limit: 25, lower dust limit: 50, optimum intensity: 20,000, maximum intensity: 30,000, minimum intensity: 10,000, pinhole: 50 μm . *Analysis parameters*: analysis method: CONTIN, weight function: 1, fitting range: $G_2(\tau)$, $G_2(\tau)_{\text{max}}$: 2, $G_2(\tau)_{\text{min}}$: 1.003. *Cell parameters*: measurement item: size, cell type: size cell, cell centre Z: 4.85 mm, cell centre X: 6.55 mm, correlator type: log, accumulation times: 100. *Dilutant parameters*: dilutant name: water, refractive index: 1.33, viscosity: 0.89, dielectric constant: 78.3.

Unless stated otherwise, polymers were purified *via* dialysis. Benzoylated dialysis tubing, with an average flat width of 9 mm, was used. Tubing capacity was approx. 10 ml/ft, pore size: 2000 MWCO, purchased from Sigma Aldrich. Reaction mixture containing synthesised polymer was pipetted into the dialysis tubing and dialysis tubing closures, purchased from Sigma Aldrich, were placed at each end. Dialysis tubing containing the solution was placed in 0.5–1.0 l deionised water for 48–72 h. Water was replaced approximately every 24 h.

Percentage conversion calculations

^1H NMR spectra were used to calculate BMA and MAA conversion. If an internal standard was used and a spectrum was obtained of the reaction mixture at 0 h it was possible to calculate the BMA and the MAA conversion from the integration values at each time point as the concentration of the standard remained constant. The internal standard (trioxane) signal was integrated as 1 on the 0 h reaction mixture spectrum, BMA (δ 5.94 br) and MAA (δ 5.97 br) signals were also integrated and recorded as 'X' values. The trioxane signal was then integrated as 1 on the reaction mixture spectrum from a later time point, BMA (δ 5.94 br) and MAA (δ 5.97 br) signals were also integrated and recorded as 'Y' values. Percentage conversion was calculated for that particular time point: $((X-Y)/X) \times 100 = \% \text{ conversion}$. Without a spectrum of the reaction mixture at 0 h it was still possible to calculate the

conversion of BMA but not the MAA conversion (**Figure 31**). It was not possible to calculate MAA conversion without the use of an internal standard as a distinct MAA polymer peak was not identifiable in the NMR spectrum.

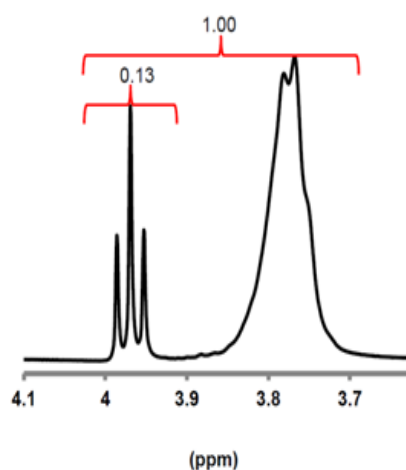
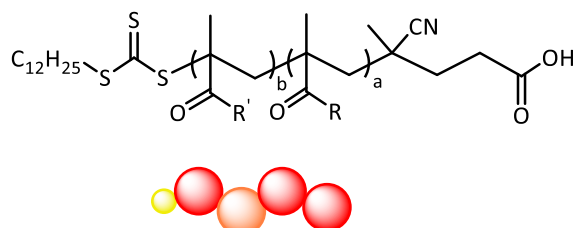


Figure 31. The triplet seen at 3.98 ppm (from the $\text{-O-CH}_2\text{-CH}_2\text{CH}_2\text{CH}_3$ protons in the BMA sections of the polymer) can be integrated in relation to the total integration of this signal plus the polymer signal seen at 3.78 ppm. If the integration of both signals is set to 1.00 then the integration of the BMA protons alone gives the percentage of BMA left in the reaction; in this example 13%. This information allows the % conversion of BMA to be calculated ($100 - 13 = 77\%$ conversion).

2.4.2 Determining copolymerisation reaction time



R: OH

R':

a = approx. 4 (9: 1 BMA: MAA), 8 (4: 1 BMA: MAA) or 13 (2: 1 BMA: MAA), b = approx. 27 (2: 1 BMA: MAA), 31 (4: 1 BMA: MAA) or 33 (9: 1 BMA: MAA).

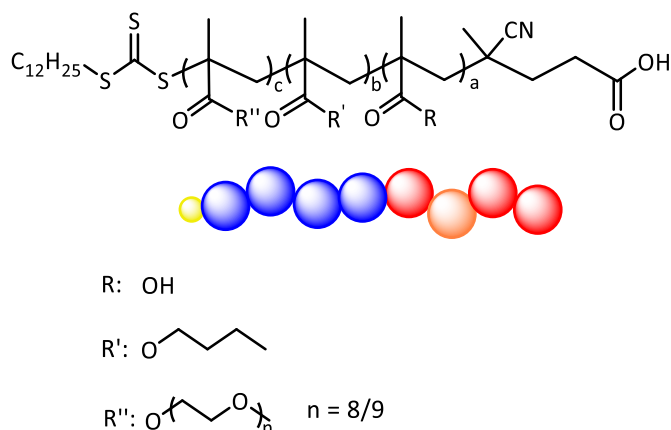
The Chemspeed parallel synthesiser was used to perform kinetic studies. Stock solutions of the RAFT agent, 4-cyano-4[(dodecylsulfanylthio carbonyl)sulfanyl] pentanoic acid, (130 mg/ml, 5 ml) and ACVA (5 mg/ml, 6 ml) were degassed *via* sparging with nitrogen for 30 min and then added to the Chemspeed parallel synthesiser. Neat BMA (5 ml), MAA (1 ml) and DMF (15 ml) were degassed using the same method and also added to the parallel synthesiser. The Chemspeed was programmed to dispense all reagents (see **Table 16** for quantities of reagents) once an inert atmosphere had been reached. Reactions were heated at 80 °C for 22 h with aliquots taken at 0, 10, 14, 18 and 22 h. **Reactor 1**, 10 h M_n (DMAc SEC):

5,500 Da, $\bar{D}_M$: 1.11, BMA conversion (^1H NMR): 96%, MAA conversion (^1H NMR): 93%. 14 h M_n (DMAc SEC): 5,600 Da, $\bar{D}_M$: 1.11, BMA conversion (^1H NMR): 96%, MAA conversion (^1H NMR): 93%. 18 h M_n (DMAc SEC): 5,600 Da, $\bar{D}_M$: 1.11, BMA conversion (^1H NMR): 96%, MAA conversion (^1H NMR): 93%. 22 h M_n (DMAc SEC): 5,700 Da, $\bar{D}_M$: 1.11, BMA conversion (^1H NMR): 97%, MAA conversion (^1H NMR): 93%. **Reactor 2**, 10 h M_n (DMAc SEC): 5,700 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 96%, MAA conversion (^1H NMR): 90%. 14 h M_n (DMAc SEC): 5,800 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 97%, MAA conversion (^1H NMR): 93%. 18 h M_n (DMAc SEC): 5,800 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 97%, MAA conversion (^1H NMR): 93%. 22 h M_n (DMAc SEC): 5,900 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 98%, MAA conversion (^1H NMR): 93%. **Reactor 3**, 10 h M_n (DMAc SEC): 5,700 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 93%, MAA conversion (^1H NMR): 87%. 14 h M_n (DMAc SEC): 5,800 Da, $\bar{D}_M$: 1.13, BMA conversion (^1H NMR): 94%, MAA conversion (^1H NMR): 89%. 18 h M_n (DMAc SEC): 5,800 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 95%, MAA conversion (^1H NMR): 89%. 22 h M_n (DMAc SEC): 5,900 Da, $\bar{D}_M$: 1.13, BMA conversion (^1H NMR): 96%, MAA conversion (^1H NMR): 89%.

	BMA	MAA	ACVA	RAFT	DMF
Reactor 1	9.76	1.08	3.20×10^{-2}	0.32	17.6
Reactor 2	8.68	2.17	3.20×10^{-2}	0.32	18.6
Reactor 3	7.23	3.61	3.20×10^{-2}	0.32	19.9

Table 16. Millimoles of reagents added to Chemspeed parallel synthesiser reactors. Reactor 1: 9: 1 BMA: MAA ratio, Reactor 2: 4: 1 BMA: MAA ratio, Reactor 3: 2: 1 BMA: MAA ratio.

2.4.3 Synthesis of quasi-block copolymers



a = approx. 4 (9: 1 BMA: MAA), 8 (4: 1 BMA: MAA) or 13 (2: 1 BMA: MAA), b = approx. 33 (9: 1 BMA: MAA), 31 (4: 1 BMA: MAA) or 27 (2: 1 BMA: MAA), c = approx. 11 (5,000 Da), 21 (10,000 Da) or 33 (15,000 Da).

Quasi-block copolymers were synthesised using the Chemspeed parallel synthesiser. Stock solutions of the RAFT agent (130 mg/ml, 8 ml) and ACVA (5 mg/ml, 27 ml) were degassed *via* sparging with nitrogen for 30 min and then added to the Chemspeed parallel synthesiser. Neat BMA (11 ml), MAA (2 ml), PEGMA (20 ml) and DMF (40 ml) were degassed using the same method and added to the parallel synthesiser. The Chemspeed parallel synthesiser dispensed BMA, MAA, RAFT agent, ACVA and DMF once an inert atmosphere had been reached (see **Table 17** for quantities). The reactors were heated for 10 h at 80 °C.

	BMA	MAA	ACVA	RAFT	DMF
Reactor 1	24.4	2.70	8.00×10^{-2}	0.81	43.9
Reactor 2	21.7	5.43	8.00×10^{-2}	0.81	46.4
Reactor 3	18.1	9.03	8.00×10^{-2}	0.81	50.4

Table 17. Millimoles of reagents added in step 1 of quasi-block copolymer synthesis.

Reactor 1, M_n (DMAc SEC): 5,500 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 96%, MAA conversion (^1H NMR): 92%. **Reactor 2**, M_n (DMAc SEC): 5,700 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 96%, MAA conversion (^1H NMR): 91%. **Reactor 3**, M_n (DMAc SEC): 5,700 Da, $\bar{D}_M$: 1.14, BMA conversion (^1H NMR): 96%, MAA conversion (^1H NMR): 92%.

Upon cooling, the reaction mixtures were each equally split three ways (into 9 total reactors) and ACVA and PEGMA (**Table 18**) were added. Reactors were then heated for a further 10 h at 80 °C. ¹H NMR and GPC aliquots were taken at 6 h.

	ACVA	PEGMA
Reactor 1	2.89×10^{-2}	2.89
Reactor 2	2.48×10^{-2}	4.96
Reactor 3	2.48×10^{-2}	7.46
Reactor 4	2.39×10^{-2}	2.39
Reactor 5	2.39×10^{-2}	4.78
Reactor 6	2.39×10^{-2}	7.18
Reactor 7	2.28×10^{-2}	2.28
Reactor 8	2.28×10^{-2}	4.57
Reactor 9	2.28×10^{-2}	6.84

Table 18. Millimoles of reagents added to reactors in step 2 of quasi-block copolymer synthesis.

Reactor 1, 6 h M_n (DMAc SEC): 7,500 Da, $\bar{D}_M$: 1.16. 10 h M_n (DMAc SEC): 7,500 Da, $\bar{D}_M$: 1.16.

Reactor 2, 6 h M_n (DMAc SEC): 8,200 Da, $\bar{D}_M$: 1.25. 10 h M_n (DMAc SEC): 8,200 Da, $\bar{D}_M$: 1.25.

Reactor 3, 6 h M_n (DMAc SEC): 2 *peaks*: 12,900, 6,100 Da, $\bar{D}_M$: 1.11, 1.10. 10 h M_n (DMAc SEC): 2 *peaks*: 11,300, 6,100 Da, $\bar{D}_M$: 1.11, 1.09. **Reactor 4**, 6 h M_n (DMAc SEC): 14,100 Da, $\bar{D}_M$: 1.30.

10 h M_n (DMAc SEC): 14,100 Da, $\bar{D}_M$: 1.31. **Reactor 5**, 6 h M_n (DMAc SEC): 13,700 Da, $\bar{D}_M$: 1.31.

10 h M_n (DMAc SEC): 13,900 Da, $\bar{D}_M$: 1.32. **Reactor 6**, 6 h M_n (DMAc SEC): 13,000 Da, $\bar{D}_M$: 1.43.

10 h M_n (DMAc SEC): 13,200 Da, $\bar{D}_M$: 1.43. **Reactor 7**, 6 h M_n (DMAc SEC): 18,300 Da, $\bar{D}_M$: 1.42.

10 h M_n (DMAc SEC): 2 *peaks*: 18,600, 6,600 Da, $\bar{D}_M$: 1.21, 1.07. **Reactor 8**, 6 h M_n (DMAc SEC): 2 *peaks*: 24,000, 6,100 Da, $\bar{D}_M$: 1.26, 1.10. 10 h M_n (DMAc SEC): 2 *peaks*: 25,400, 6,000 Da, $\bar{D}_M$:

1.25, 1.10. **Reactor 9**, 6 h M_n (DMAc SEC): 2 *peaks*: 23,100, 6,000 Da, $\bar{D}_M$: 1.25, 1.10. 10 h M_n (DMAc SEC): 2 *peaks*: 23,300, 6,100 Da, $\bar{D}_M$: 1.25, 1.09.

2.4.4 Optimising reaction conditions

Effect of initiator (ACVA) concentration on rate of reaction

This study was performed using six reactors in the Chemspeed automated parallel synthesiser. BMA (5 ml), MAA (1 ml), DMF (35 ml) and stock solutions of RAFT agent (120 mg/ml, 3 ml) and ACVA (10 mg/ml, 2 ml) in DMF were degassed *via* sparging with nitrogen for 30 min and then loaded into the Chemspeed. The Chemspeed parallel synthesiser dispensed BMA, MAA, RAFT agent, ACVA and DMF once an inert atmosphere had been reached (see **Table 19** for quantities). The reactors were heated for 8 h at 80 °C. ^1H NMR and GPC aliquots were taken at 1, 2, 4, 6 and 8 h.

	BMA	MAA	ACVA	RAFT	DMF
Reactor 1	4.50	0.50	1.49×10^{-2}	0.15	22.5
Reactor 2	4.00	1.00	1.42×10^{-2}	0.14	22.9
Reactor 3	3.33	1.67	1.34×10^{-2}	0.13	23.6
Reactor 4	4.50	0.50	7.43×10^{-3}	0.15	22.5
Reactor 5	4.00	1.00	7.12×10^{-3}	0.14	22.9
Reactor 6	3.33	1.67	6.72×10^{-3}	0.13	23.6

Table 19. Millimoles of reagents added to reactors in study of the effect of initiator (ACVA) concentration on monomer conversion in copolymerisation reaction.

Reactor 1, 1 h M_n (DMAc SEC): 3,100 Da, $\bar{D}_M$: 1.09, BMA conversion (^1H NMR): 35%. 2 h M_n (DMAc SEC): 3,700 Da, $\bar{D}_M$: 1.10, BMA conversion (^1H NMR): 52%. 4 h M_n (DMAc SEC): 4,300 Da, $\bar{D}_M$: 1.11, BMA conversion (^1H NMR): 73%. 6 h M_n (DMAc SEC): 4,600 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 77%. 8 h M_n (DMAc SEC): 4,700 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 80%. **Reactor 2**, 1 h M_n (DMAc SEC): 3,500 Da, $\bar{D}_M$: 1.10, BMA conversion (^1H NMR): 40%. 2 h M_n (DMAc SEC): 4,200 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 58%. 4 h M_n (DMAc SEC): 4,800 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 75%. 6 h M_n (DMAc SEC): 5,100 Da, $\bar{D}_M$: 1.13, BMA conversion (^1H NMR): 79%. 8 h M_n (DMAc SEC): 5,200 Da, $\bar{D}_M$: 1.13, BMA conversion (^1H NMR): 81%. **Reactor 3**, 1 h M_n (DMAc SEC): 3,700 Da, $\bar{D}_M$: 1.10, BMA conversion (^1H NMR): 38%. 2 h M_n (DMAc SEC): 4,300 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 57%. 4 h M_n (DMAc SEC): 5,000 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 73%. 6 h M_n (DMAc SEC): 5,200 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 77%. 8 h M_n (DMAc SEC): 5,200 Da, $\bar{D}_M$: 1.14, BMA conversion (^1H NMR): 79%. **Reactor 4**, 1 h M_n (DMAc SEC): 2,800 Da, $\bar{D}_M$: 1.07, BMA conversion (^1H NMR): 26%. 2 h M_n (DMAc SEC): 3,200 Da, $\bar{D}_M$: 1.09, BMA conversion (^1H NMR): 37%. 4 h M_n (DMAc SEC): 3,600 Da, $\bar{D}_M$: 1.11, BMA conversion (^1H NMR): 55%. 6 h M_n (DMAc SEC): 3,800 Da, $\bar{D}_M$: 1.11, BMA conversion (^1H NMR): 59%. 8 h M_n (DMAc SEC): 4,000 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 60%. **Reactor 5**, 1 h M_n (DMAc SEC): 3,000 Da, $\bar{D}_M$: 1.07, BMA conversion (^1H NMR): 24%. 2 h M_n (DMAc SEC): 3,100 Da, $\bar{D}_M$: 1.09, BMA conversion (^1H NMR): 28%. 4 h M_n (DMAc SEC): 3,400 Da, $\bar{D}_M$: 1.11, BMA conversion (^1H NMR): 40%. 6 h M_n (DMAc SEC): 3,500 Da, $\bar{D}_M$: 1.11, BMA conversion (^1H NMR): 42%. 8 h M_n (DMAc SEC): 3,600 Da, $\bar{D}_M$: 1.11, BMA conversion (^1H NMR): 43%. **Reactor 6**, 1 h M_n (DMAc SEC): 3,300 Da, $\bar{D}_M$: 1.09, BMA conversion (^1H NMR): 28%. 2 h M_n (DMAc SEC): 3,400 Da, $\bar{D}_M$: 1.09, BMA conversion (^1H NMR): 32%. 4 h M_n (DMAc SEC): 3,700 Da, $\bar{D}_M$: 1.11, BMA conversion (^1H NMR): 41%. 6 h M_n (DMAc SEC): 3,700 Da, $\bar{D}_M$: 1.11,

BMA conversion (^1H NMR): 43%. 8 h M_n (DMAc SEC): 3,800 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 44%.

Azobis(cyclohexanecarbonitrile) as initiator

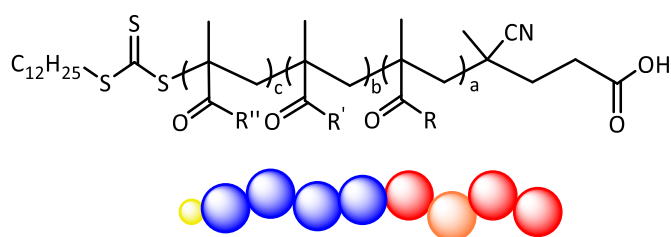
Three reactions were performed using the Chemspeed automated parallel synthesiser. BMA (2 ml), MAA (1 ml), DMF (5 ml) and stock solutions of RAFT agent (120 mg/ml, 2 ml) and ACHN (10 mg/ml, 1.5 ml) in DMF were degassed *via* sparging with nitrogen for 30 min and then loaded into the Chemspeed. The Chemspeed dispensed BMA, MAA, RAFT agent, ACHN and DMF once an inert atmosphere had been reached (see for **Table 20** quantities). The reactors were heated for 12 h at 80 °C.

	BMA	MAA	ACHN	RAFT	DMF
Reactor 1	4.50	0.50	1.49×10^{-2}	0.15	22.5
Reactor 2	4.00	1.00	1.42×10^{-2}	0.14	22.9
Reactor 3	3.33	1.67	1.34×10^{-2}	0.13	23.6

Table 20. Millimoles of reagents used in trial of ACHN initiator.

Reactor 1, M_n (DMAc SEC): 6,200 Da, $\bar{D}_M$: 1.13, BMA conversion (^1H NMR): 80%. **Reactor 2**, M_n (DMAc SEC): 7,700 Da, $\bar{D}_M$: 1.18, BMA conversion (^1H NMR): 87%. **Reactor 3**, M_n (DMAc SEC): 8,000 Da, $\bar{D}_M$: 1.20, BMA conversion (^1H NMR): 88%.

Quasi-block copolymer synthesis using ACHN



R: OH

R': O-CH₂-CH₂-CH₂-CH₃

R'': O-(CH₂-CH₂-O)_n n = 8/9

a = approx. 4 (5,000 Da), 5 (7,500 Da), 7 (10,000 Da) or 11 (15,000 Da). b = approx. 33 (5,000 Da), 49 (7,500 Da), 66 (10,000 Da) or 99 (15,000 Da). c = approx. 11 (5,000 Da), 21 (10,000 Da), 16 (7,500 Da) or 33 (15,000 Da).

A library of seven quasi-block copolymers was synthesised using seven reactors in the Chemspeed automated parallel synthesiser. BMA (6 ml), MAA (1 ml), PEGMA (7 ml), DMF (35 ml) and stock solutions of RAFT agent (120 mg/ml, 3 ml) and ACHN (10 mg/ml, 3 ml) in DMF were degassed *via* sparging with nitrogen for 30 min and then loaded into the Chemspeed. The Chemspeed parallel synthesiser dispensed BMA (0.72 ml, 4.50 mmol), MAA (0.04 ml, 0.50 mmol), RAFT agent, ACHN and DMF (1.74 ml, 2.25×10^{-2} mmol) once an inert atmosphere had been reached (see **Table 21** for quantities). Reactors were heated for 12 h at 85 °C. Upon cooling ACHN and PEGMA (see **Table 21**) were added. Reactors were then heated for a further 12 h at 85 °C.

	ACHN	RAFT	PEGMA	ACHN (2)
Reactor 1	1.49×10^{-2}	0.15	1.57	7.43×10^{-3}
Reactor 2	1.49×10^{-2}	0.15	3.14	7.43×10^{-3}
Reactor 3	1.49×10^{-2}	0.15	4.70	7.43×10^{-3}
Reactor 4	9.62×10^{-3}	9.62×10^{-2}	1.52	4.81×10^{-3}
Reactor 5	7.12×10^{-3}	7.12×10^{-2}	0.75	3.56×10^{-3}
Reactor 6	7.12×10^{-3}	7.12×10^{-2}	1.50	3.56×10^{-3}
Reactor 7	4.68×10^{-3}	4.68×10^{-2}	0.49	2.34×10^{-3}

Table 21. Millimoles of reagents used to synthesise quasi-block copolymers using ACHN.

Reactor 1, M_n (DMAc SEC): 5,600 Da, $\bar{D}_M$: 1.13, BMA conversion (^1H NMR): 85%, MAA conversion (^1H NMR): 74%. **Reactor 2**, M_n (DMAc SEC): 5,600 Da, $\bar{D}_M$: 1.12, BMA conversion (^1H NMR): 83%, MAA conversion (^1H NMR): 72%. **Reactor 3**, M_n (DMAc SEC): 5,300 Da, $\bar{D}_M$: 1.13, BMA conversion (^1H NMR): 83%, MAA conversion (^1H NMR): 71%. **Reactor 4**, M_n (DMAc SEC): 8,200 Da, $\bar{D}_M$: 1.15, BMA conversion (^1H NMR): 88%, MAA conversion (^1H NMR): 78%. **Reactor 5**, M_n (DMAc SEC): 10,400 Da, $\bar{D}_M$: 1.17, BMA conversion (^1H NMR): 89%, MAA conversion (^1H NMR): 77%. **Reactor 6**, M_n (DMAc SEC): 10,200 Da, $\bar{D}_M$: 1.17, BMA conversion (^1H NMR): 85%, MAA conversion (^1H NMR): 71%. **Reactor 7**, M_n (DMAc SEC): 14,900 Da, $\bar{D}_M$: 1.17, BMA conversion (^1H NMR): 86%, MAA conversion (^1H NMR): 73%.

Determining reaction time and ACHN concentration

Fourteen quasi-block copolymers were synthesised using reactors in the Chemspeed automated parallel synthesiser. BMA (11 ml), MAA (1 ml), PEGMA (12 ml), DMF (35 ml) and stock solutions of RAFT agent (120 mg/ml, 6 ml) and ACHN (10 mg/ml, 4 ml) in DMF were degassed *via* sparging with nitrogen for 30 min and then loaded into the Chemspeed. The Chemspeed dispensed BMA (0.72 ml, 4.50 mmol), MAA (0.04 ml, 0.50 mmol), RAFT agent,

ACHN and DMF (1.74 ml, 22.5 mmol) once an inert atmosphere had been reached (see **Table 22** for quantities) and reactors were heated for 9 h at 85 °C. Aliquots were taken at 9 h. Upon cooling ACHN and PEGMA (**Table 22**) were added. Reactors were then heated for a further 7 h at 85 °C.

	ACHN	RAFT	PEGMA	ACHN (2)
Reactor 1	1.49×10^{-2}	0.15	1.57	7.43×10^{-3}
Reactor 2	1.49×10^{-2}	0.15	3.14	7.43×10^{-3}
Reactor 3	1.49×10^{-2}	0.15	4.70	7.43×10^{-3}
Reactor 4	9.62×10^{-3}	9.62×10^{-2}	1.52	4.81×10^{-3}
Reactor 5	7.12×10^{-3}	7.12×10^{-2}	0.75	3.56×10^{-3}
Reactor 6	7.12×10^{-3}	7.12×10^{-2}	1.50	3.56×10^{-3}
Reactor 7	4.68×10^{-3}	4.68×10^{-2}	0.49	2.34×10^{-3}
Reactor 8	1.11×10^{-2}	0.15	1.57	7.43×10^{-3}
Reactor 9	1.11×10^{-2}	0.15	3.14	7.43×10^{-3}
Reactor 10	1.11×10^{-2}	0.15	4.70	7.43×10^{-3}
Reactor 11	7.22×10^{-3}	9.62×10^{-2}	1.52	4.81×10^{-3}
Reactor 12	5.34×10^{-3}	7.12×10^{-2}	0.75	3.56×10^{-3}
Reactor 13	5.34×10^{-3}	7.12×10^{-2}	1.50	3.56×10^{-3}
Reactor 14	3.51×10^{-3}	4.68×10^{-2}	0.49	2.34×10^{-3}

Table 22. Millimoles of reagents and stock solutions used in synthesis of quasi-block copolymers with reduced reaction time and ACHN concentration.

Reactor 1, M_n (DMAc SEC): 10,400 Da, $\bar{D}_M$: 1.26, BMA conversion (^1H NMR): 74%, MAA conversion (^1H NMR): 60%, PEGMA conversion (DMAc SEC): 110%. **Reactor 2**, M_n (DMAc SEC): 12,000 Da, $\bar{D}_M$: 1.33, BMA conversion (^1H NMR): 72%, MAA conversion (^1H NMR): 61%, PEGMA conversion (DMAc SEC): 71%. **Reactor 3**, M_n (DMAc SEC): 14,200 Da, $\bar{D}_M$: 1.39, BMA conversion (^1H NMR): 70%, MAA conversion (^1H NMR): 55%, PEGMA conversion (DMAc SEC): 62%. **Reactor 4**, M_n (DMAc SEC): 12,600 Da, $\bar{D}_M$: 1.27, BMA conversion (^1H NMR): 66%, MAA conversion (^1H NMR): 51%, PEGMA conversion (DMAc SEC): 80%. **Reactor 5**, M_n (DMAc SEC): 14,100 Da, $\bar{D}_M$: 1.28, BMA conversion (^1H NMR): 61%, MAA conversion (^1H NMR): 45%, PEGMA conversion (DMAc SEC): 119%. **Reactor 6**, M_n (DMAc SEC): 16,200 Da, $\bar{D}_M$: 1.29, BMA conversion (^1H NMR): 55%, MAA conversion (^1H NMR): 37%, PEGMA conversion (DMAc SEC): 88%. **Reactor 7**, M_n (DMAc SEC): 16,000 Da, $\bar{D}_M$: 1.27, BMA conversion (^1H NMR): 49%, MAA conversion (^1H NMR): 33%, PEGMA conversion (DMAc SEC): 139%. **Reactor 8**, M_n (DMAc SEC): 10,400 Da, $\bar{D}_M$: 1.32, BMA conversion (^1H NMR): 69%, MAA conversion (^1H NMR): 54%, PEGMA conversion (DMAc SEC): 107%. **Reactor 9**, M_n (DMAc SEC): 12,700 Da, $\bar{D}_M$: 1.59, BMA conversion (^1H NMR): 69%, MAA conversion (^1H NMR): 53%, PEGMA conversion (DMAc SEC): 76%. **Reactor 10**, M_n (DMAc SEC): 15,400 Da, $\bar{D}_M$: 1.36, BMA conversion (^1H NMR): 63%, MAA

conversion (^1H NMR): 46%, PEGMA conversion (DMAc SEC): 72%. **Reactor 11**, M_n (DMAc SEC): 12,700 Da, $\bar{D}_M$: 1.27, BMA conversion (^1H NMR): 55%, MAA conversion (^1H NMR): 36%, PEGMA conversion (DMAc SEC): 90%. **Reactor 12**, M_n (DMAc SEC): 14,100 Da, $\bar{D}_M$: 1.30, BMA conversion (^1H NMR): 58%, MAA conversion (^1H NMR): 41%, PEGMA conversion (DMAc SEC): 123%. **Reactor 13**, M_n (DMAc SEC): 15,400 Da, $\bar{D}_M$: 1.33, BMA conversion (^1H NMR): 60%, MAA conversion (^1H NMR): 56%, PEGMA conversion (DMAc SEC): 77%. **Reactor 14**, M_n (DMAc SEC): 14,600 Da, $\bar{D}_M$: 1.29, BMA conversion (^1H NMR): 41%, MAA conversion (^1H NMR): 23%, PEGMA conversion (DMAc SEC): 102%.

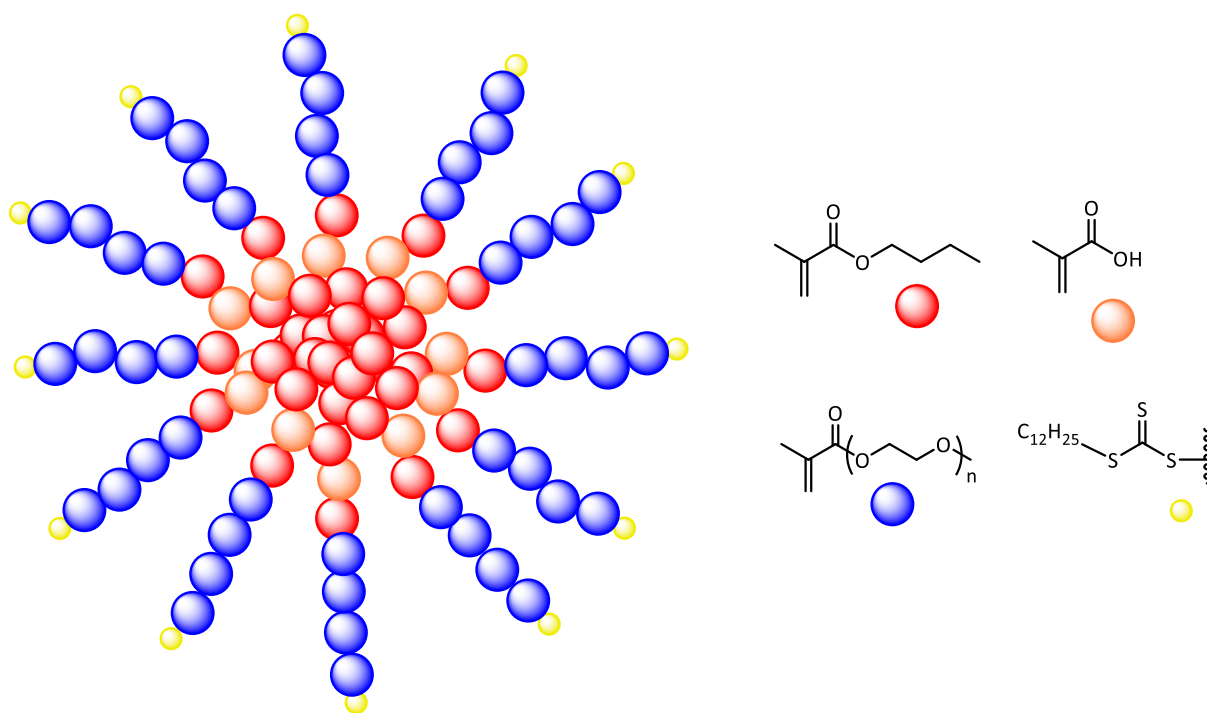
Bench synthesised polymers

BMA, MAA, ACHN (3.63 mg, 0.015 mmol), RAFT agent 4-cyano-4[(dodecylsulfenylthio carbonyl)sulfanyl] pentanoic acid (59.96 mg, 0.15 mmol) and trioxane (approximately 10 mg) were dissolved in DMF (2.5 ml, 32.3 mmol). The quantities of monomers added can be found in **Table 23**. The solution was then transferred to a Young's tap Schlenk flask and the mixture was degassed *via* the freeze-pump-thaw method (3 x 20 min cycles). After the solution had thawed and warmed to room temperature it was heated at 85°C for 9 h. Upon completion of the reaction the mixture was allowed to cool and then pure-block copolymers were purified *via* dialysis then lyophilised and redissolved in DMF before PEGMA (0.69 mL, 1.57 mmol), ACHN (1.8 mg, 0.007 mmol) and DMF (1 mL) were added to the reaction vessel. In the synthesis of quasi-block copolymers the PEGMA and ACHN were added into the cooled reaction mixture after the first step of the reaction. The reaction mixture was degassed again, *via* the freeze-pump-thaw method (3 x 20 min cycles) and then allowed to thaw and warm to room temperature before being heated at 85°C for 7 h. Upon cooling the quasi-block copolymers were purified *via* dialysis and isolated *via* lyophilisation.

Ratio of BMA: MAA	Quantity of BMA used		Quantity of MAA used	
9: 1	0.72 mL	4.50 mmol	0.04 mL	0.50 mmol
4: 1	0.67 mL	4.21 mmol	0.09 mL	1.05 mmol
2: 1	0.59 mL	3.72 mmol	0.16 mL	1.86 mmol

Table 23. Quantities of BMA and MAA used to make 5,000 Da BMA-co-MAA copolymers.

2.4.5 Self-assembly of micelles

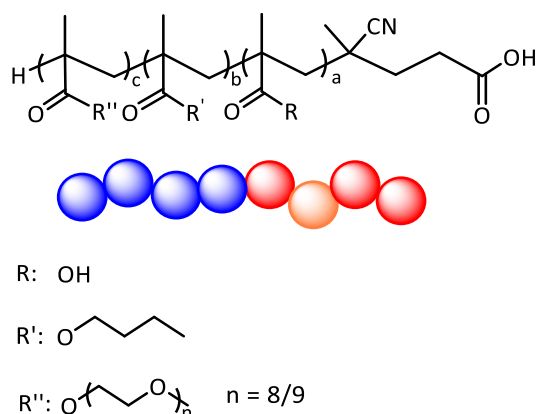


Micelles were self-assembled in deionised water. Polymer samples (1 mg) were dissolved in water (2 ml) to give micelle solutions. Vortex and ultrasonic mixing was required to dissolve the majority of polymers. Higher molecular weight polymers were generally less soluble. DLS was used to measure the micelle diameter, five measurements were acquired per sample and averaged to provide the final result (**Table 24**).

Polymer M_n	1	2	3	4	5	Average
10,400	39	40	38	38	32	37
12,000	51	52	51	51	52	51
14,200	50	50	48	51	51	50
12,600	47	50	48	54	51	50
14,100	51	55	53	54	55	54
16,200	58	60	57	59	60	59
16,100	66	67	67	64	65	66
12,700	53	57	52	59	62	57
15,400	52	49	54	53	53	52
14,600	175	166	165	174	170	170
8,000	16	20	16	15	16	16
6,500	36	36	35	36	36	36
5,400	23	23	23	23	34	25
3,800	23	25	26	26	24	25
5,900	26	27	27	27	27	27
6,400	43	41	40	39	38	40
6,700	44	42	42	42	40	42
9,500	26	20	26	25	26	25
8,900	54	41	50	49	47	48
7,300	26	25	25	27	24	25
11,900	126	128	121	123	124	124
17,900	173	182	140	182	173	170
25,900	147	149	141	142	142	144
13,600	146	153	156	157	156	154
24,800	128	131	126	126	123	127

Table 24. DLS results for polymers self-assembled into micelles. M_n values given are in Da, micelle measurements are the micelle diameter in nm. Five measurements were acquired per sample and averaged to provide the final result.

2.4.6 Removal of RAFT agent



a = approx. 7 (10,000 Da, 9: 1 BMA: MAA), 15 (20,000 Da, 9: 1 BMA: MAA) or 22 (30,000 Da, 9: 1 BMA: MAA), 27 (10,000 Da, 2: 1 BMA: MAA), or 81 (30,000 Da, 2: 1 BMA: MAA). b = approx. 66 (10,000 Da, 9: 1 BMA: MAA), 132 (20,000 Da, 9: 1 BMA: MAA), 198 (30,000 Da, 9: 1

BMA: MAA), 54 (10,000 Da, 2: 1 BMA: MAA), 108 (20,000 Da, 2: 1 BMA: MAA) or 162 (30,000 Da, 2: 1 BMA: MAA). c = 11 (5,000 Da) or 32 (15,000 Da).

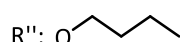
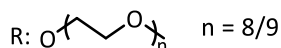
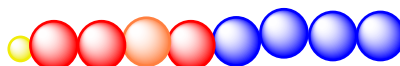
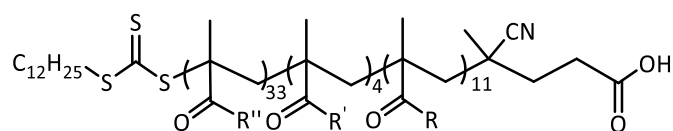
RAFT agent end groups were removed from six polymer samples (see **Table 25**). The same method was used for all six reactions, with the exception of the purification step; two different purification techniques were used. Block copolymer BMA-co-MAA-*b*-PEGMA (0.013 mmol), ACHN (1.6 mg, 6.5 μ mol) and ethyl piperidine hypophosphite (12 mg, 0.065 mmol) were dissolved in acetonitrile. The solution was degassed *via* nitrogen purging (30 min). The mixture was heated in a microwave reactor for 2 h at 100 °C, 150 W. Upon completion of the reaction the polymer was purified by one of the following purification methods:

- 1) Solvent was removed to give a clear, colourless gel. The product was dissolved in a solution of water and THF. The cloudy white solution was washed with diethyl ether and DCM which resulted in the formation of a fine white precipitate in the THF/H₂O layer. As the solid was too fine to isolate *via* filtration, the THF was removed under reduced pressure and then water was removed *via* lyophilisation.
- 2) Solvent, by products and unreacted ethylpiperidine hypophosphite were removed *via* dialysis in water. The product was dried *via* lyophilisation.

Sample	BMA: MAA ratio	M_n (Da)	$\mathcal{D}_M$	Purification technique
1	9: 1	11,900	1.13	1
2	9: 1	17,900	1.14	2
3	9: 1	25,900	1.13	1
4	2: 1	11,700	1.19	2
5	2: 1	13,600	1.18	1
6	2: 1	24,800	1.12	1

Table 25. Purification of polymers used in RAFT end group removal study. M_n and $\mathcal{D}_M$ determined via GPC.

2.4.7 'Reverse' quasi-block copolymer synthesis



PEGMA (0.69 ml, 1.6 mmol), ACHN (3.6 mg, 0.015 mmol), RAFT agent 4-cyano-4[(dodecylsulfenylthio carbonyl)sulfanyl] pentanoic acid (60 mg, 0.15 mmol) and trioxane (10 mg, 0.11 mmol), were dissolved in DMF (2.5 ml). The solution was then transferred to a Young's tap Schlenk flask and the mixture was degassed *via* the freeze-pump-thaw method (3 x 20 min cycles). After the solution had thawed and warmed to room temperature it was heated at 85 °C for 12 h. Upon completion of the reaction the mixture was allowed to cool before BMA (0.72 ml, 4.5 mmol), MAA (0.04 ml, 0.5 mmol), ACHN (1.8 mg, 0.007 mmol) and DMF (1 ml) were added to the reaction vessel. The mixture was then degassed again *via* the freeze-pump-thaw method (3 x 20 min cycles) and allowed to thaw and warm to room temperature before being heated at 85 °C for 9 h. Upon cooling, the quasi-block copolymers were purified *via* dialysis in water. Water was removed using lyophilisation. M_n (DMAc SEC): 7,400 Da, D_M : 1.22, PEGMA conversion (DMAc SEC): 124%, BMA conversion (^1H NMR): 79%.

Chapter III

Adaptation of Micelles in Preparation
for Biological Studies

3.1 Introduction

Micelles are easily self-assembled in water from amphiphilic block copolymers and have great potential for *in vivo* drug delivery and other applications such as imaging.^{157,170,204} The synthesis of a library of block-copolymer micelles was described in **Chapter II**. Before micelles could be tested *in vitro* they needed to be stabilised to prevent disassembly at high dilution and tagged so that their location can be determined once administered.

3.1.1 Crosslinking

Self-assembled micelles are dynamic systems with polymer chains in the particle continuously exchanging with single chains in solution.¹⁷¹ In terms of micelle stability both thermodynamic and kinetic stability have to be considered. Kinetic stability concerns the interchange of polymers between micelle and free polymer chains and the disassembly of the micelle.¹⁷¹ Thermodynamic stability affects the self-assembly of the micelle particle; the more hydrophobic the core, the more stable the micelle will be and therefore the lower the CMC. The encapsulation of a hydrophobic drug within the core can also have an effect on the thermodynamic stability of the particle.⁷ Lee *et al.* found that higher levels of encapsulated hydrophobic drug correlated with longer release times as the micelles were slower to disassemble.²⁰⁵

Tuning the stability of a micelle is particularly important in drug delivery; the micelle needs to be stable enough to remain intact *in vitro* and *in vivo* as it travels to its target destination. The concentration of micelles required *in vitro* will likely be lower than the CMC and after administration *in vivo*, micelles will be extremely diluted as they travel in the blood stream.²⁰⁶ The tuning of micelle stability is one of the major issues preventing current delivery systems from reaching the clinical trial stage.^{171,169} Micelles have to be highly stabilised to remain intact at low concentrations and yet it is also vital that the micelles disassemble when they reach the target destination so that the drug is released.²⁰⁷

Crosslinking the chains within a dynamic micelle stabilises the micelle, transforming it into a static structure that will not disassemble at low concentrations. Crosslinking is essential for ensuring the stability of the micelle once it is removed from the solution it was originally assembled in. The disassembly of a micelle before it reaches its target destination would

result in a completely ineffective treatment as the drug will not be delivered. The size of micelle particles is generally unaffected by the crosslinking process, however, there are some reports in the literature of crosslinking steps having an effect on micelle size.^{175,176,208,209} The effect of crosslinking on micelle size can be easily determined for individual systems using dynamic light scattering (DLS).

Micelle particles can either be core crosslinked or shell crosslinked as illustrated in **Figure 32**. Both methods have advantages and disadvantages. The application of the micelle should be considered when determining what kind of crosslinking methodology should be used. There are many examples of core crosslinking in the literature, with three main methodologies available. The first method is to incorporate side chains into the polymer block which makes up the core of the micelle. These side chains can either be polymerised with side chains on an adjacent polymer or connected using a linker (e.g. a diamine linker) once the micelle is assembled. The second option for core crosslinking involves the addition of a reactive functional group to the end of the hydrophobic polymer block, which can subsequently be polymerised with the ends of other polymer chains in the micelle. It is also possible to internalise small molecules inside the micelle core and then polymerise them but this method is less commonly used.²¹⁰ The first method has been proven to be effective in many systems.^{176,211} For example, Liu *et al.* incorporated cinnamoyl side groups into the core forming block of their micelles which were then polymerised using a photochemical reaction.^{212,213} Examples of the second method of core crosslinking include work by Iijima *et al.*, who successfully crosslinked the core of their poly(ethylene glycol)-*b*-polylactide copolymer micelles using a methacryloyl end group, crosslinked *via* radical polymerisation.²¹⁰

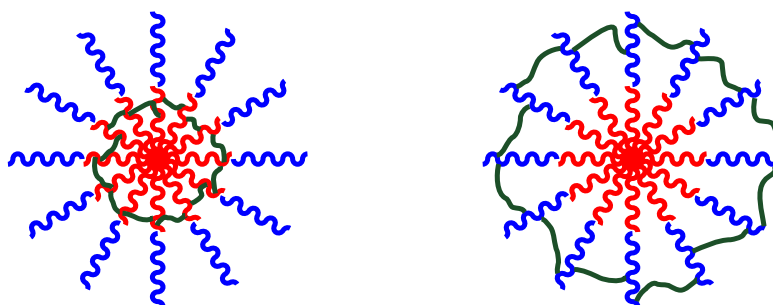


Figure 32. Representations of core crosslinking (left) and shell crosslinking (right).

Shuai *et al.* core-crosslinked triblock copolymers using radical polymerisation and found that crosslinked versions of the micelles had higher paclitaxel load efficacy than uncrosslinked micelles.¹⁵⁴ The core crosslinking was achieved by polymerising the poly(ϵ -caprolactone) block that made up the core of the micelles *via* radical polymerisation. Generally, core crosslinking has a negative impact on encapsulation efficacy, it can interfere with the encapsulation of the cargo as the free volume of the core is reduced.^{206,210} For micelle particles designed to transport a cargo within their core, shell crosslinking is the more practical option.²⁰⁸

Shell crosslinking has become increasingly popular since it was first reported by Wooley *et al.* in 1996.²¹⁴ Crosslinking a micelle's shell (also known as the corona) usually involves the incorporation of a reactive or polymerisable functional group in the shell block of the polymer. Adjacent chains can then be polymerised or connected using a linker unit with difunctionality. A wide range of chemistry has been used to shell crosslink, including click chemistry, photocrosslinking, Michael addition, ionic crosslinking and carbodiimide coupling reactions.²¹⁵ Responsive crosslinking is becoming common as researchers try to address the need for micelles to disassemble once they reaches their target. Both pH responsive and temperature responsive crosslinking has been successful.^{10,216} pH responsive polymers use variations in pH in the body, e.g. in certain cell types or in the digestive system, to facilitate micelle disassembly and drug release.²¹⁷ Other types of responsive crosslinking utilise high levels of specific enzymes in target cells. For example, McCormick *et al.* synthesised an α -methoxy poly(ethylene oxide)-*b*-poly(*N*-(3-aminopropyl) methacrylamide)- β -poly(2-diisopropylamino) ethyl methacrylate) triblock copolymer with a reversible disulphide crosslinker.¹⁸⁷ Disulfide crosslinkers can be cleaved using glutathione or enzymes such as nicotinamide adenine dinucleotide phosphate-oxidase, which has been found to be activated in cancerous cells.^{218,219}

Pascual and Monteiro have reported the successful crosslinking of P(NIPAM-*b*-DMA) (poly(*N*-isopropylacrylamide *N,N*-dimethylacrylamide)) block copolymers *via* the incorporation of a *N*-acryloyloxysuccinimide (NAS) block into the polymer.²⁰⁹ The NAS blocks on adjacent polymer chains were crosslinked with a primary diamine, resulting in stable, shell crosslinked micelle particles. Wooley *et al.* also had success using this method to crosslink poly(*N*-(acryloyloxy)succinimide-co-*N*-acryloylmorpholine)-*b*-poly(L-lactic acid) block copolymer

micelles.²²⁰ NAS-diamine crosslinking can be reversed by peptidases which break down amide bonds.²²⁰

The best way to determine if a crosslinking step has been successful varies between systems. Analytical techniques such as NMR, UV-Vis and HPLC can be used if a functional group which has a distinctive peak/signal is involved in the crosslinking process. Either the disappearance or appearance of the identified peak can be used to quantify the extent of crosslinking.^{207,175,221} Piogé *et al.* crosslinked the core of diblock copolymer micelles by incorporating methacrylate groups at the end of each polymer chain. The methacrylate groups were then polymerised using light and a photoinitiator, once the micelles had been assembled, to crosslink the core. It was possible to show 100% conversion of the methacrylate double bonds to confirm the success of the crosslinking step.¹⁷⁵ Stenzel *et al.* used HPLC to determine the rate and extent of the crosslinking step in their poly(poly(ethylene glycol) methyl ether methacrylate)-*b*-poly(methyl methacrylate-co-poly(3-isopropenyl- α,α -dimethylbenzyl isocyanate)) block copolymer system.²⁰⁷ The micelles were crosslinked using a diamine linker and pendant isocyanate groups on poly(3-isopropenyl- α,α -dimethylbenzyl isocyanate) units incorporated into the hydrophobic block. HPLC was used to monitor the consumption of 1,8-diaminooctane. Other, less technical methods for determining the success of crosslinking include diluting micelle solutions below the CMC and cooling solutions below the lowest critical solution temperature (LCST) to see if micelles remain intact.^{154,222} Crosslinking of micelles can also be verified by dissolving micelles in a solvent which causes uncrosslinked micelles to dissociate.^{151,223} This is a popular technique to use when NMR or other analytical spectra are complicated and specific peaks are hard to identify.

Shell crosslinking *via* the incorporation of NAS into the hydrophilic block of BMA-co-MAA-*b*-PEGMA, subsequently followed by the introduction of a diamine linker was used in this project. Shell crosslinking was chosen to allow for optimum encapsulation within the micelle core. A NAS-diamine linking system was selected as macrophages are known to contain peptidases, which facilitate the breakdown of amide bonds and will therefore reverse crosslinking of the micelle and result in the release of encapsulated cargo.^{224–226}

3.1.2 Fluorescent labelling

There has been much research, and many examples to-date, of fluorescently labelled nanoparticles. Labelling of nanoparticles allows drug delivery systems to be tracked *in vivo* and *in vitro* and so is essential for analysis of these systems. **Figure 33** shows some examples of commonly used fluorescent dyes. Many dyes can be purchased as monomers which makes them easy to incorporate into polymer systems, either as part of the initial polymer synthesis or as an additional block, examples can be found in **Figure 34**.

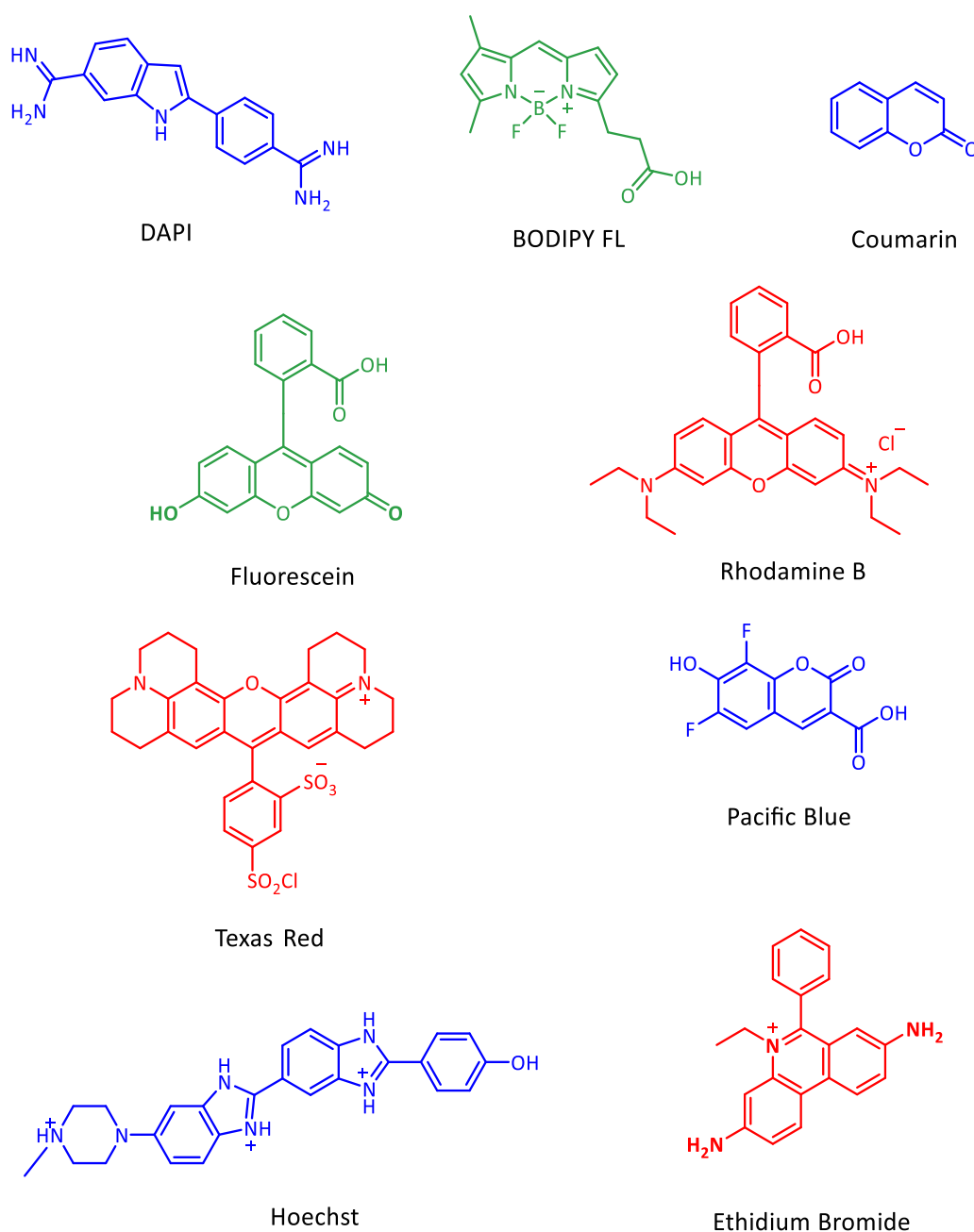
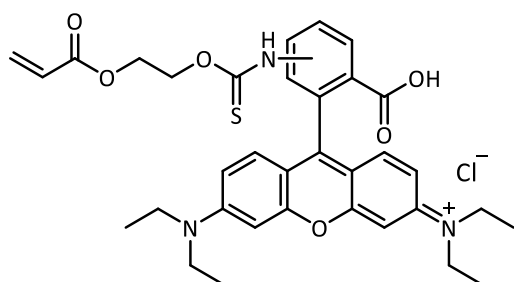
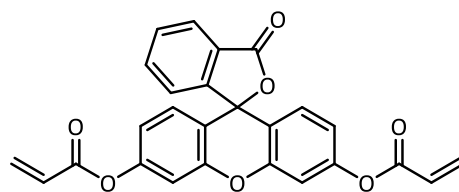


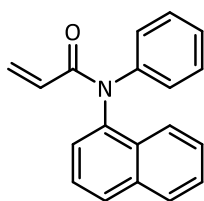
Figure 33. Common fluorescent dyes.



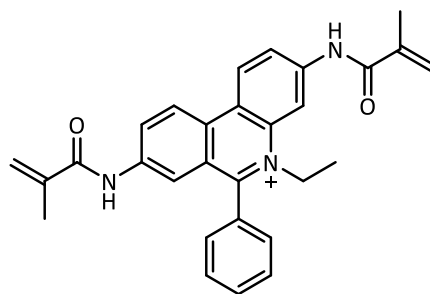
Acryloxyethyl thiocarbamoyl Rhodamine B



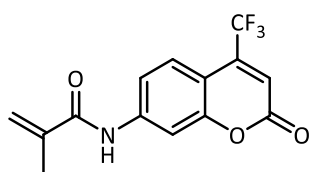
Fluorescein *O,O'*-diacrylate



N-(1-Naphthyl)-*N*-phenylacrylamide



Ethidium bromide-*N,N'*-bismethacrylamide



7-[4-(Trifluoromethyl)coumarin]methacrylamide

Figure 34. Examples of fluorescent monomers which can easily be polymerised to fluorescently label polymer systems.

Fluorescent monomers can be incorporated into micelle particles at different stages of the synthesis, either in the initial block synthesis, where the fluorescent tag might be incorporated as part of a copolymer block, or the fluorescent monomer can be polymerised onto the end of the polymer chain after the synthesis of the rest of the block copolymer. A third option is to add the fluorescent tag after the micelle has been assembled. A large variety of fluorescent monomers are available; there are compatible options for all popular polymerisation techniques, including RAFT, ATRP and nitroxide-mediated radical polymerisation.^{227,228,106} Fluorescent tags can also be attached to RAFT agents prior to the polymerisation. Farinha *et al.* detail an example in a paper describing the synthesis of a

poly(*N*-decylacrylamide)-*b*-poly(*N,N*-diethylacrylamide) block copolymer using a RAFT agent with a phenanthrene group.²²⁹

In this work a rhodamine B functionalised monomer, methacryloxyethyl thiocarbamoyl rhodamine B (MRhB), **Figure 35**, was incorporated into the hydrophilic block of BMA-co-MAA block copolymers using RAFT polymerisation. Rhodamine B is a phenyl xanthene which is not easily bleached or sensitive to light.

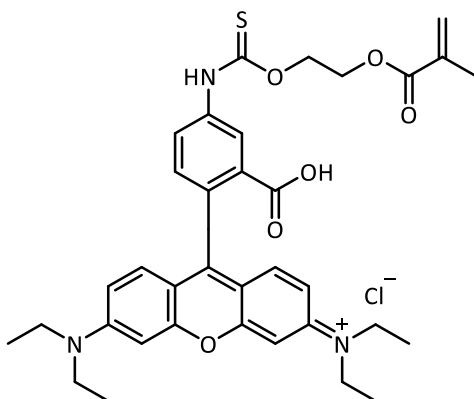


Figure 35. Methacryloxyethyl thiocarbamoyl rhodamine B (MRhB).

3.2 Results and Discussion

3.2.1 Stability of micelles

Micelles used in *in vitro* studies need to be stable at very low concentrations. To determine the stability of micelles prepared in this work studies were performed investigating the formation of micelles at low concentrations and the effect of high temperatures on micelle formation and size. The Pendant drop method and DLS were both used to investigate micelle formation at low concentrations. Micelles selected for crosslinking and fluorescence labelling in preparation for use in *in vitro* studies are displayed in **Table 26**.

Polymer synthesis	Polymer M_n (Da)	$\bar{D}_M$	Micelle diameter (nm)
QB	6,900	1.13	16 $\pm$ 5
QB	7,300 [^]	1.34	24 $\pm$ 2
PB	3,800	1.18	25 $\pm$ 11
PB	5,400	1.17	25 $\pm$ 11
QB	7,300 [^]	1.17	25 $\pm$ 3
PB	5,900	1.19	27 $\pm$ 2
QB	6,500	1.24	36 $\pm$ 2
QB	7,800	1.27	37 $\pm$ 11
PB	6,400	1.18	40 $\pm$ 5
PB	6,700	1.12	42 $\pm$ 4
QB	7,700	1.27	47 $\pm$ 9
PB	4,100	1.15	48 $\pm$ 13
QB	14,200 [*]	1.39	49 $\pm$ 9
QB	12,600 [*]	1.27	50 $\pm$ 16
QB	15,400	1.33	52 $\pm$ 10
QB	14,100 [*]	1.28	54 $\pm$ 15
QB	12,700	1.27	57 $\pm$ 20
QB	16,200 [*]	1.29	59 $\pm$ 25
QB	16,100 [*]	1.27	66 $\pm$ 30
QB	7,400	1.21	120 $\pm$ 16
QB	10,000	1.16	124 $\pm$ 64
QB	25,900 [^]	1.13	144 $\pm$ 72
QB	17,900 [^]	1.14	170 $\pm$ 10

Table 26. Summary of micelles selected for crosslinking and fluorescent labelling in preparation for cellular uptake studies. QB: quasi-block copolymer, PB: pure-block copolymer. The majority of M_n and $\bar{D}_M$ values calculated using GPC (THF SEC) at CSIRO, Clayton, Australia, [^] M_n and $\bar{D}_M$ values calculated using GPC (THF SEC) at Imperial College London, ^{*} M_n and $\bar{D}_M$ values calculated using GPC (DMAc SEC) at CSIRO, Clayton, Australia. All GPC details can be found in section **3.4.1**.

Effect of concentration on micelle formation

The Pendant drop method and dynamic light scattering (DLS) were used to determine the critical micelle concentration (CMC) and the effect of concentration on micelle formation. In the Pendant drop method, the interfacial tension of micelle solutions of different concentrations are measured and plotted to determine the CMC. In a plot of interfacial tension versus concentration a change in gradient as the concentration is lowered indicates the CMC. Results from the pendant drop method suggested the CMC was approximately $1.3 \times 10^{-5} \text{ mol/dm}^3$, see **Figure 36**. DLS results, displayed in **Table 27**, showed that micelles were still formed at concentrations as low as $7.8 \times 10^{-7} \text{ mol/dm}^3$, although micelles formed at concentrations lower than $1.3 \times 10^{-6} \text{ mol/dm}^3$ were considerably larger than the micelles formed at higher concentrations. This is clearly highlighted in **Figure 37**. DLS and the Pendant drop method results were in correspondence, it was therefore concluded that stable micelles form at concentrations $> 1.3 \times 10^{-5} \text{ mol/dm}^3$.

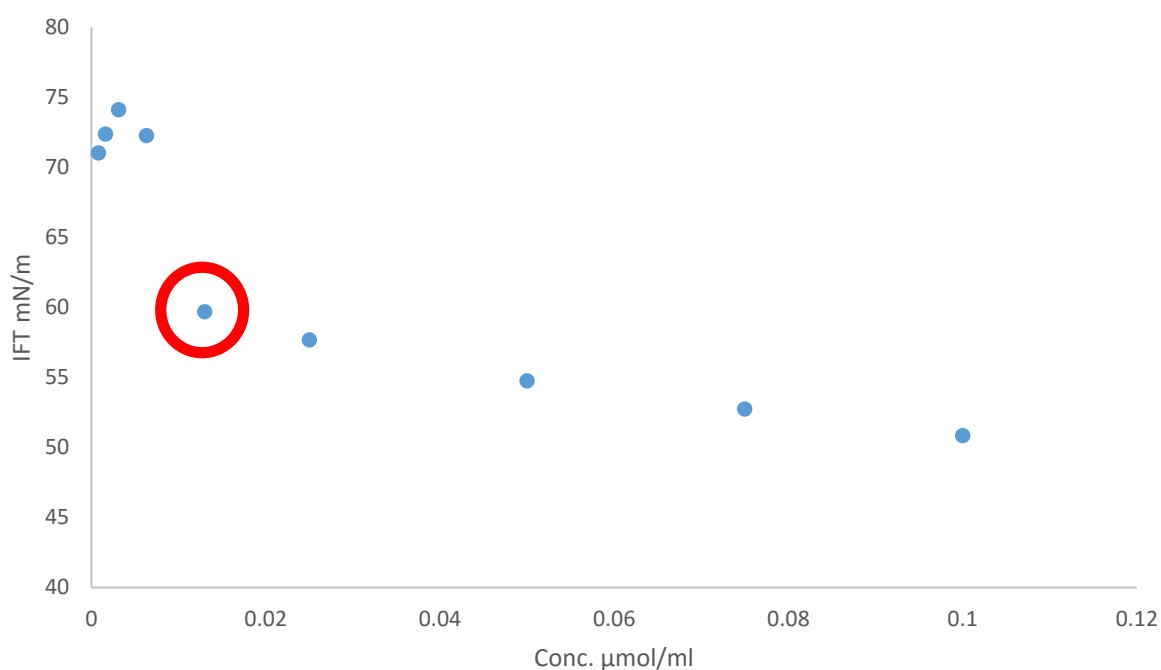


Figure 36. Pendant drop method results for 10,400 Da BMA-co-MAA-*b*-PEGMA block copolymer (4,900 Da BMA-co-MAA block, 5,500 Da PEGMA block, 9: 1 BMA: MAA). Plot of interfacial tension (IFT) measurement against polymer concentration. Change in gradient at $1.3 \times 10^{-2} \pm 6.0 \times 10^{-3} \mu\text{mol/ml}$ (circled in red) indicated the CMC.

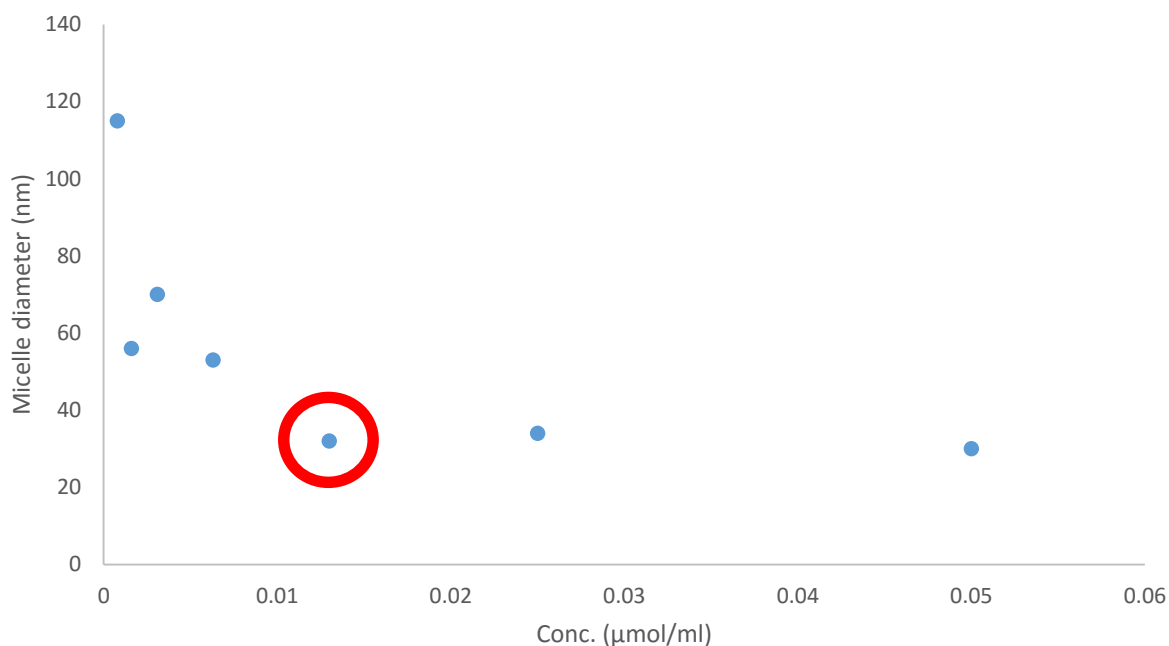


Figure 37. Plot showing micelle size versus polymer concentration for a 10,400 Da BMA-co-MAA-*b*-PEGMA block copolymer (4,900 Da BMA-co-MAA block, 5,500 Da PEGMA block, 9: 1 BMA: MAA). Change in gradient clearly seen at $1.3 \times 10^{-2} \pm 6.0 \times 10^{-3}$ μmol/ml (circled in red), indicated the CMC.

Sample concentration (μmol/ml)	Micelle diameter (nm)	Range in diameter (nm)
5.0×10^{-2}	30	27-34
2.5×10^{-2}	34	31-37
1.3×10^{-2}	32	28-36
6.3×10^{-3}	53	41-64
3.1×10^{-3}	70	61-80
1.6×10^{-3}	56	42-70
7.8×10^{-3}	115	53-178

Table 27. DLS results in a comparison between sample concentration and micelle diameter for a 10,400 Da BMA-co-MAA-*b*-PEGMA block copolymer (4,900 Da BMA-co-MAA block, 5,500 Da PEGMA block, 9: 1 BMA: MAA). Micelle diameter remains constant when the concentration is $1.3 \times 10^{-2} \pm 6.0 \times 10^{-3}$ μmol/ml or above. Micelles formed at concentrations lower than $1.3 \times 10^{-2} \pm 6.0 \times 10^{-3}$ μmol/ml were larger and the range in diameter increased.

Effect of heat on micelle formation

To test the stability of the micelles at high temperatures, solutions of micelles in water were heated from 25 °C to 70 °C in increments of 5 °C. Micelle diameter was measured using DLS. Results are displayed in **Table 28** and **Figure 38**. Micelles remained intact at high temperatures and their size was not affected. Micelle diameters were re-measured upon cooling, results

showed that heating the particles to high temperatures had no significant effect on the micelle size.

Temperature (°C)	Micelle A diameter (nm)	Micelle B diameter (nm)	Micelle C diameter (nm)
25	16	25	36
30	17	26	35
35	17	24	42
40	17	25	36
45	18	23	34
50	17	23	34
55	18	23	40
60	17	23	37
65	18	23	35
70	17	23	35

Table 28. Effect of temperature on micelle size. Micelle solutions used at a concentration of 0.5 mg/ml. Range of micelle diameters shown in parentheses. Micelle A: quasi triblock copolymer BMA-co-MAA-*b*-PEGMA-*b*-NAS-co-MRhB. Micelle B: quasi diblock copolymer, BMA-co-MAA-*b*-PEGMA-co-NAS. Micelle C: pure triblock copolymer, BMA-co-MAA-*b*-PEGMA-*b*-NAS-co-MRhB.

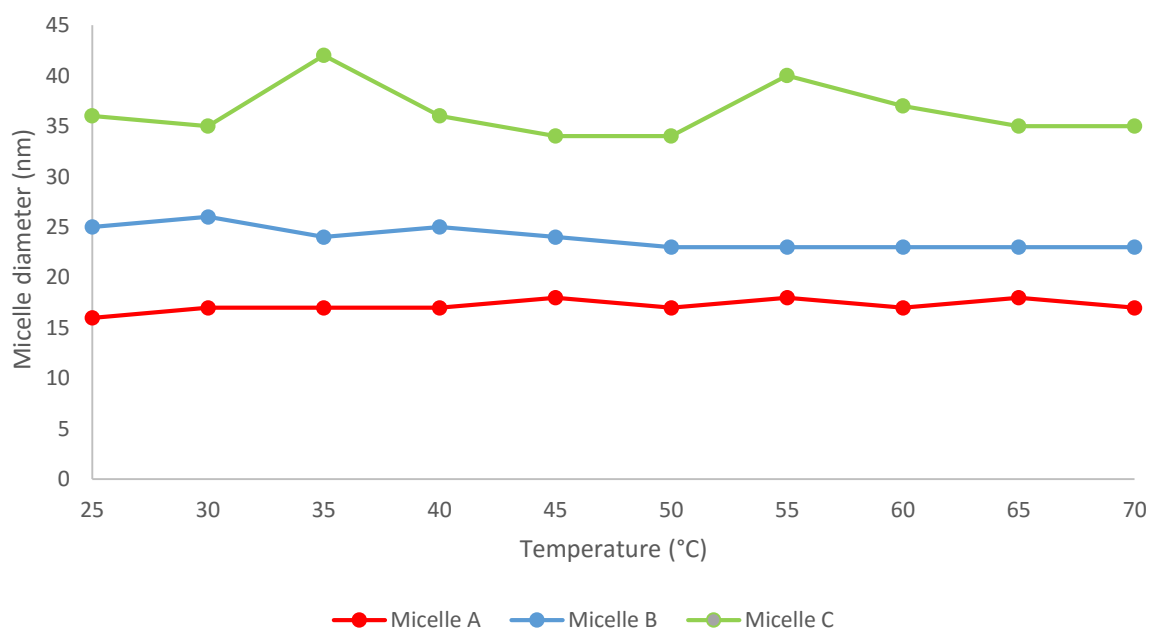


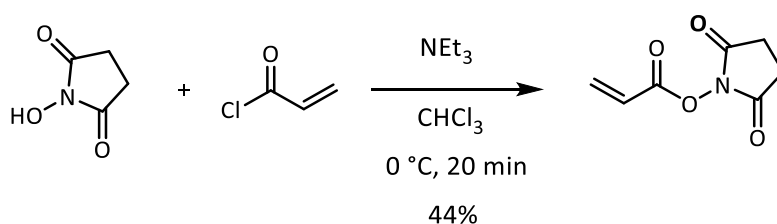
Figure 38. Micelle diameter when solutions were heated > 25 °C in increments of 5 °C. Micelle A: quasi triblock copolymer BMA-co-MAA-*b*-PEGMA-*b*-NAS-co-MRhB. Micelle B: quasi diblock copolymer, BMA-co-MAA-*b*-PEGMA-co-NAS. Micelle C: pure triblock copolymer, BMA-co-MAA-*b*-PEGMA-*b*-NAS-co-MRhB.

3.2.2 Determining crosslinking methodology

Although micelles were stable at high temperatures and still formed at low concentrations, they were crosslinked to further increase their thermodynamic stability. Crosslinking also increases the kinetic stability of the micelle as the system is no longer dynamic; the polymer chains are locked in place by the crosslinker and do not exchange with free chains in solution. The concentration of micelle solutions needed for *in vitro* studies is lower than those tested (approximately $2.0 \times 10^{-3} \mu\text{mol/ml}$). Due to the sensitivity of the DLS equipment it was not possible to investigate micelle formation at concentrations in the range used for cellular studies.

As the purpose of synthesised micelles was to encapsulate cargo within the acidic, hydrophobic core, it was decided that core crosslinking should be avoided as this would likely have an effect on the encapsulation properties of the core. Shell crosslinking was attempted *via* incorporation of NAS into the PEGMA block of the BMA-co-MAA-*b*-PEGMA block copolymer (**Scheme 12**), followed by crosslinking of the NAS units using a primary diamine (**Scheme 14**). An advantage of the chosen crosslinking method is that NAS is hydrophilic so incorporation into the PEGMA chain will not interfere with micelle formation.

Synthesis of NAS



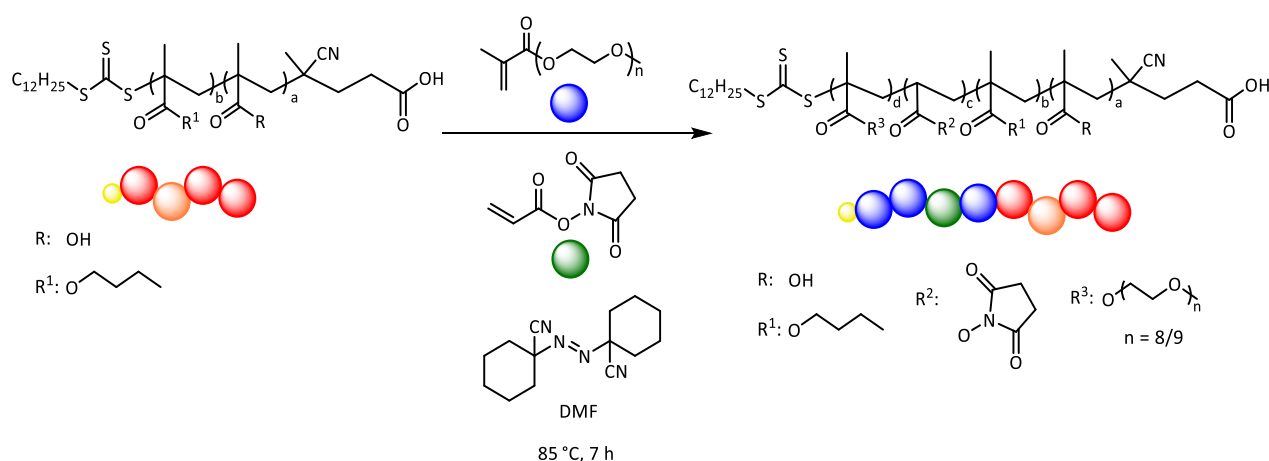
Scheme 11. Synthesis of *N*-acryloxysuccinimide (NAS).

NAS was synthesised *via* a nucleophilic acyl substitution of acryloyl chloride using *N*-hydroxysuccinimide under basic conditions (**Scheme 11**). The literature procedure described by Pollak *et al.* was followed.²³⁰ The product was obtained in a moderate yield (44%).

Incorporation of NAS into hydrophilic block

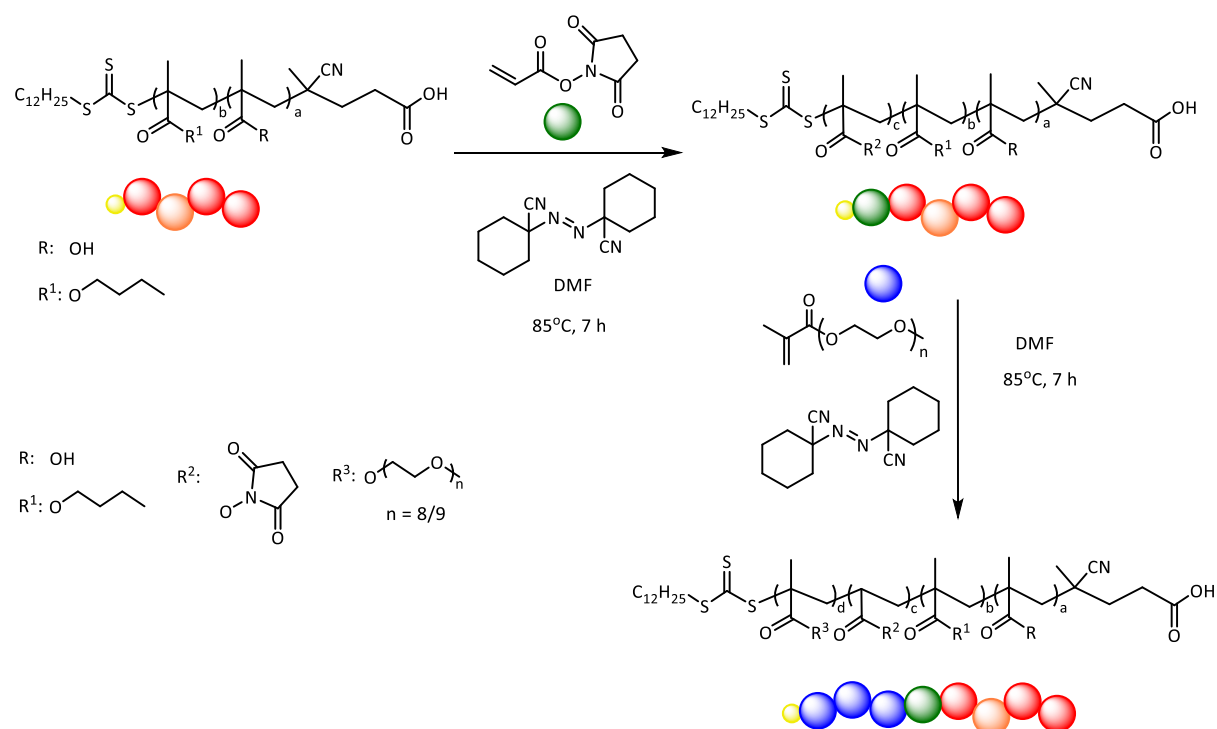
Youk *et al.* advise that when crosslinking units are positioned near the outside of a particle's shell inter-micelle crosslinking can occur.²³¹ For this reason triblock copolymers (**method B**) were synthesised for comparison with diblock copolymers (**method A**). In **method B** the BMA-co-MAA block was synthesised first. A NAS block was inserted and then finally the PEGMA block (**Scheme 13** and **Scheme 14**). Using a triblock copolymer should prevent crosslinking between micelles as none of the units being crosslinked are situated in the outer shell of the particle. As well as varying the order of monomers in the polymer the ratio of PEGMA: NAS was varied. Ratios of 10: 3 and 2: 1, PEGMA: NAS were used, triblock and diblock copolymers were both synthesised with different PEGMA: NAS ratios for comparison.

Method A



Scheme 12. NAS was added to the block copolymerisation reaction of PEGMA to give a BMA-co-MAA-*b*-PEGMA-co-NAS diblock copolymer. The PEGMA: NAS ratio was 10: 1. The ratio of BMA: MAA was 9: 1 and both copolymer blocks had a target MW of approximately 5,000 Da. a = approx. 4, b = approx. 33, c = approx. 11, d = approx. 3.

Method B



Scheme 13. Conditions for the kinetic study of the addition of a NAS block to copolymer BMA-co-MAA. Followed by the addition of PEGMA block to block copolymer BMA-co-MAA-*b*-NAS to give triblock copolymer BMA-co-MAA-*b*-NAS-*b*-PEGMA. *a* = approx. 4, *b* = approx. 33, *c* = approx. 11, *d* = approx. 3.

A kinetic study was carried out to determine the optimum reaction time for the addition of the NAS block (**Scheme 13**). The reaction was run for 7 h, aliquots were taken at 0, 1.5, 3, 4, 5, 6 and 7 h. The percentage NAS conversion was calculated using the ¹H NMR spectra with trioxane as an internal standard. The disappearance of the NAS double bond signal (δ 6.6 ppm) was measured in relation to the trioxane peak; it was possible to calculate the NAS conversion from the integration values at each time point as the concentration of the internal standard remained constant. Results are displayed in **Table 29** and **Figure 39**. The optimum reaction time was found to be 6 h.

Reaction time (h)	% NAS conversion
0	0
1.5	42
3	72
4	91
5	94
6	96
7	96

Table 29. Time compared to percentage NAS conversion in a kinetic study to determine the optimum reaction time for the addition of a NAS block to an existing BMA-co-MAA copolymer. NAS conversion calculated from integration against internal standard (trioxane) in NMR spectra.

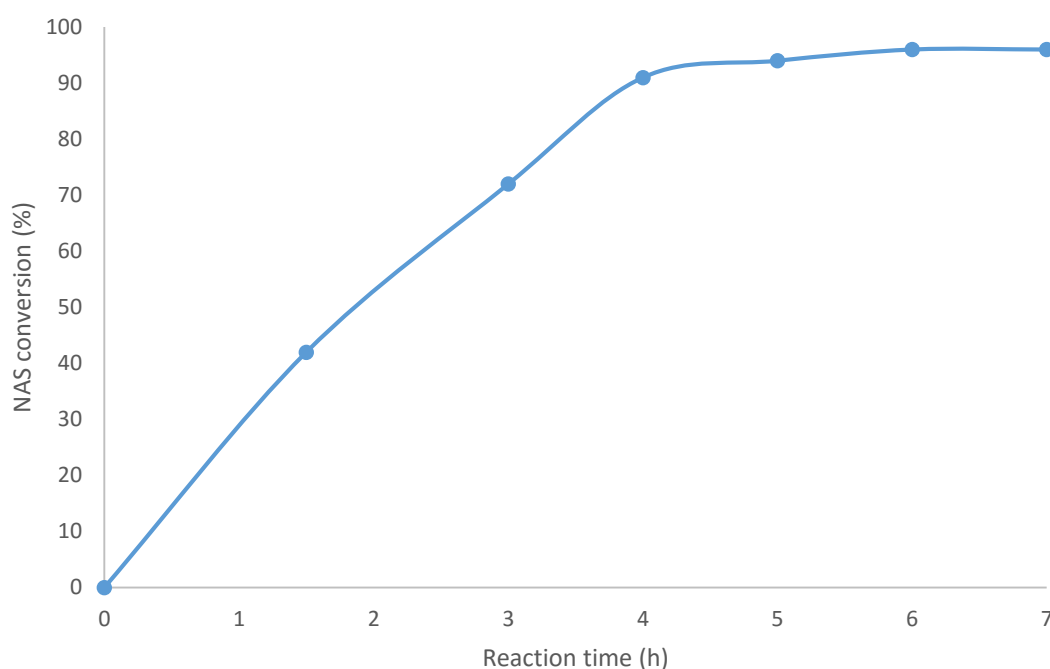
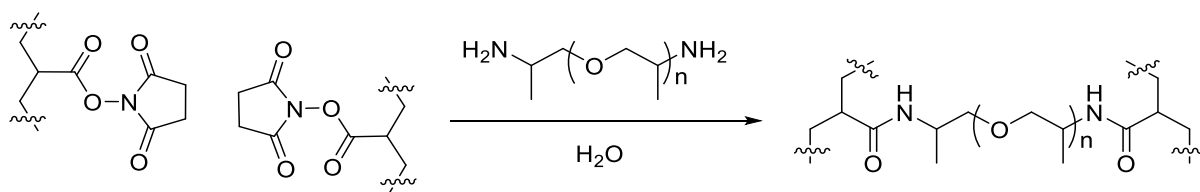


Figure 39. Plot of % NAS conversion versus reaction time.



Scheme 14. In self-assembled micelles NAS groups in the hydrophilic block were crosslinked using polyetheramines, $n = 2/3$

Initial DLS analysis of micelles formed from block copolymers with incorporated NAS, suggested that smaller micelles are formed compared to when there is no NAS in the block

copolymer (10,000 Da BMA-co-MAA-*b*-PEGMA block copolymers form micelles with an average diameter c.a. 30 nm). The triblock copolymer with a PEGMA: NAS ratio of 2: 1 was an exception to this as it formed micelles approximately 6 times larger than the other NAS-incorporated polymers, although it is not known why. NAS-incorporated polymers were assembled into micelles and crosslinked using a diamine crosslinker in water (**Scheme 14**).²⁰⁹

Comparison between crosslinked di- and triblock copolymer micelles showed the particles to be of similar sizes, indicating that inter-micelle crosslinking was not an issue when diblock copolymer micelles were crosslinked.

Polymer	PEGMA: NAS ratio	M_n (Da)	Micelle diameter (nm)
Diblock copolymer BMA-co-MAA- <i>b</i> -PEGMA-co-NAS	10: 3	9,100	22 ±6
Triblock copolymer BMA-co-MAA- <i>b</i> -NAS- <i>b</i> -PEGMA	10: 3	9,900	23 ±3
Diblock copolymer BMA-co-MAA- <i>b</i> -PEGMA- <i>b</i> -NAS	2: 1	8,700	19 ±2
Triblock copolymer BMA-co-MAA- <i>b</i> -NAS- <i>b</i> -PEGMA	2: 1	8,500	118 ±3

Table 30. Synthesised di- and triblock copolymers with incorporated NAS. The target MW of all the block copolymers was 10 kDa (5 kDa hydrophobic portion and 5 kDa hydrophilic portion). The ratio of BMA: MAA was kept at 9: 1.

Analysis of crosslinked micelles

The success of crosslinking is commonly determined either by lowering the concentration below the CMC to see if the micelle remains intact or by increasing the temperature above the CMT and determining whether the micelle is still intact.^{154,222} Due to the stability of uncrosslinked micelles at high temperatures and low concentrations, the success of the crosslinking step was instead determined *via* a comparison of the behaviour of the crosslinked micelles and the non-crosslinked micelles in a range of solvents. Diblock and triblock copolymers with 10: 3 PEGMA: NAS ratios were analysed to determine if the percentage of NAS was high enough to increase micelle stability when the particles were crosslinked.

Polymer	Solvent				
	Water (nm)	Hexane (nm)	THF (nm)	Isopropanol (nm)	Toluene (nm)
BMA-co-MAA- <i>b</i> -PEGMA-co-NAS	27	No micelles formed	No micelles formed	No micelles formed	No micelles formed
Crosslinked BMA-co-MAA- <i>b</i> -PEGMA-co-NAS	49	Sample insoluble	90	96	No micelles formed
BMA-co-MAA- <i>b</i> -NAS- <i>b</i> -PEGMA	22	No micelles formed	No micelles formed	No micelles formed	No micelles formed
Crosslinked BMA-co-MAA- <i>b</i> -NAS- <i>b</i> -PEGMA	31	Sample insoluble	130	119	No micelles formed

Table 31. Table showing the behaviour of polymer samples in a variety of solvents. Average diameter of micelle particles formed given in nm, measured using DLS.

The difference in results between the crosslinked and non-crosslinked micelles suggests that the crosslinking step was successful (**Table 31**). Although some particles were seen in the solutions of crosslinked micelles in toluene they were not consistently observed and no one size could be measured repeatedly. It was concluded that the crosslinked micelles were unstable in toluene due to the non-polar nature of the solvent. Overall the results indicated that 10: 3, PEGMA: NAS is an appropriate ratio to prepare stably crosslinked micelles and therefore this ratio was used in further crosslinking reactions.

3.2.3 Fluorescent labelling of micelles

Micelles were fluorescently labelled to allow tracking *in vitro*. Rhodamine B was selected as the fluorescent label as commercially available methacrylate monomer version of rhodamine B, methacryloxyethyl thiocarbamoyl rhodamine B (**Figure 35**), can be easily polymerised.⁴¹⁻⁴³ The excitation wavelength for rhodamine B is 554 nm and the emission wavelength is 627 nm.

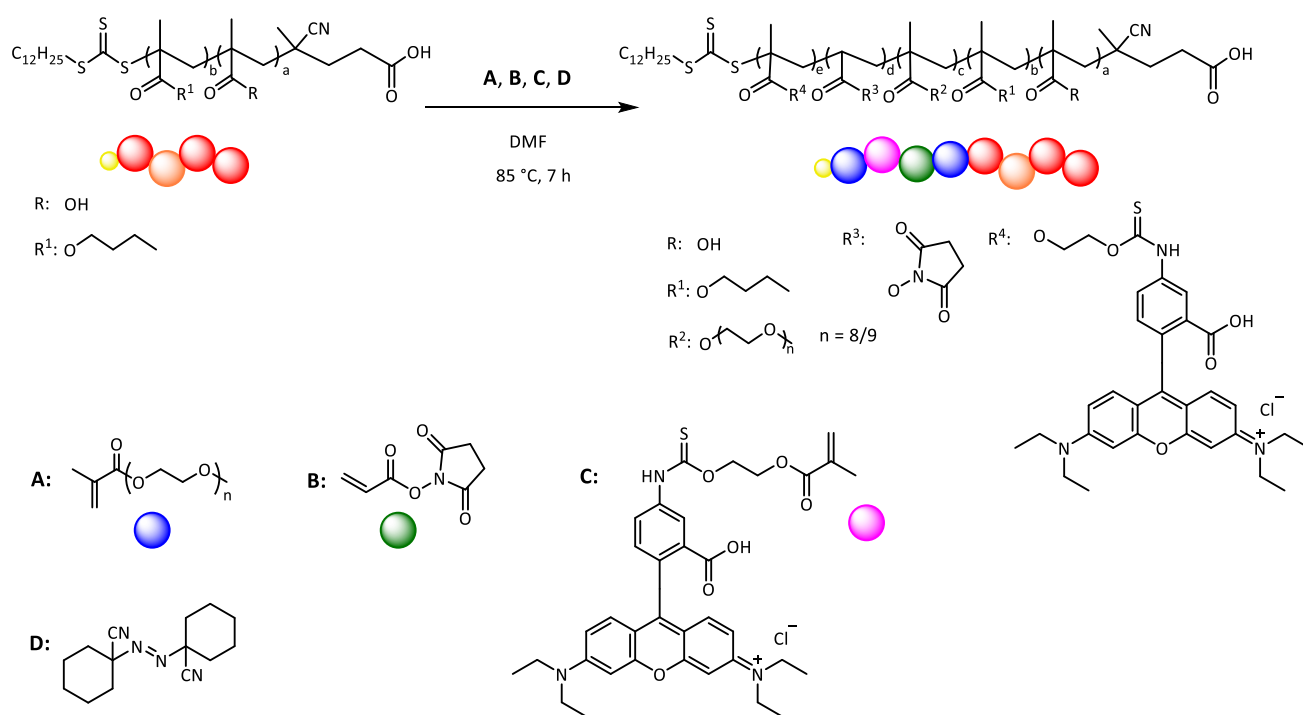
Incorporation of rhodamine B into polymer chains

Rhodamine B was incorporated into the hydrophilic head group of polymers prior to self-assembly. Incorporation was achieved *via* RAFT polymerisation of methacryloxyethyl thiocarbamoyl rhodamine B (MRhB). Four different variations in polymer composition were trialled when incorporating MRhB. The syntheses of the different variations are outlined in

Methods A, B, C and D. **Method A** involved the synthesis of a rhodamine diblock copolymer: BMA-co-MAA-*b*-NAS-co-PEGMA-co-MRhB. In method B the triblock copolymer, BMA-co-MAA-*b*-PEGMA-co-NAS-*b*-MRhB was synthesised. Method C was the synthesis of a rhodamine triblock copolymer: BMA-co-MAA-*b*-PEGMA-*b*-NAS-co-MRhB and method D, the synthesis of a rhodamine tetrablock copolymer: BMA-co-MAA-*b*-PEGMA-*b*-NAS-*b*-MRhB. The micelles assembled from the three polymer compositions were compared to determine if polymer composition had an effect on micelle formation (**Table 32**).

Method A

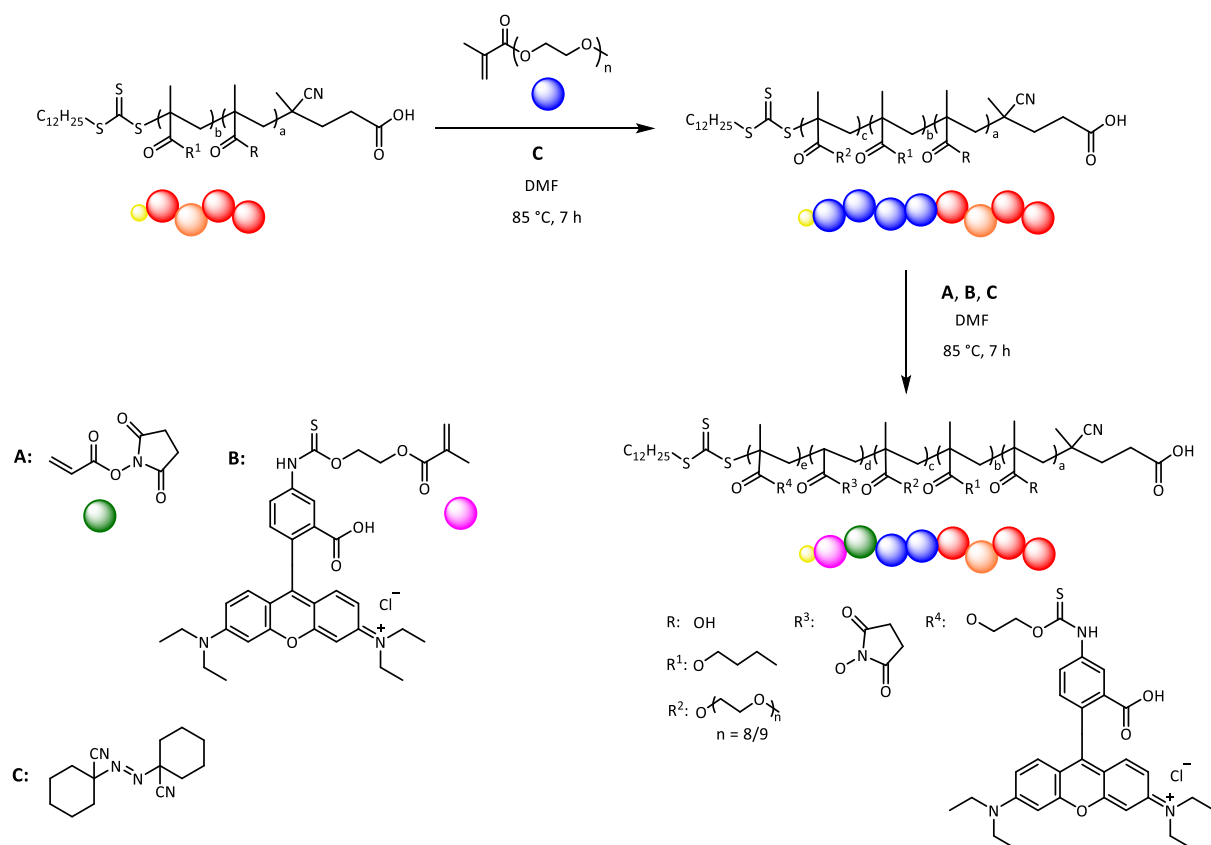
The BMA-co-MAA macroRAFT agent was extended in one step to the diblock copolymer: BMA-co-MAA-*b*-PEGMA-co-NAS-co-MRhB.



Scheme 15. Block copolymerisation of BMA-co-MAA macroRAFT agent to form rhodamine labelled diblock copolymer. a = approx. 4, b = approx. 33, c = approx. 11, d = approx. 3, e = approx. 0.05.

Method B

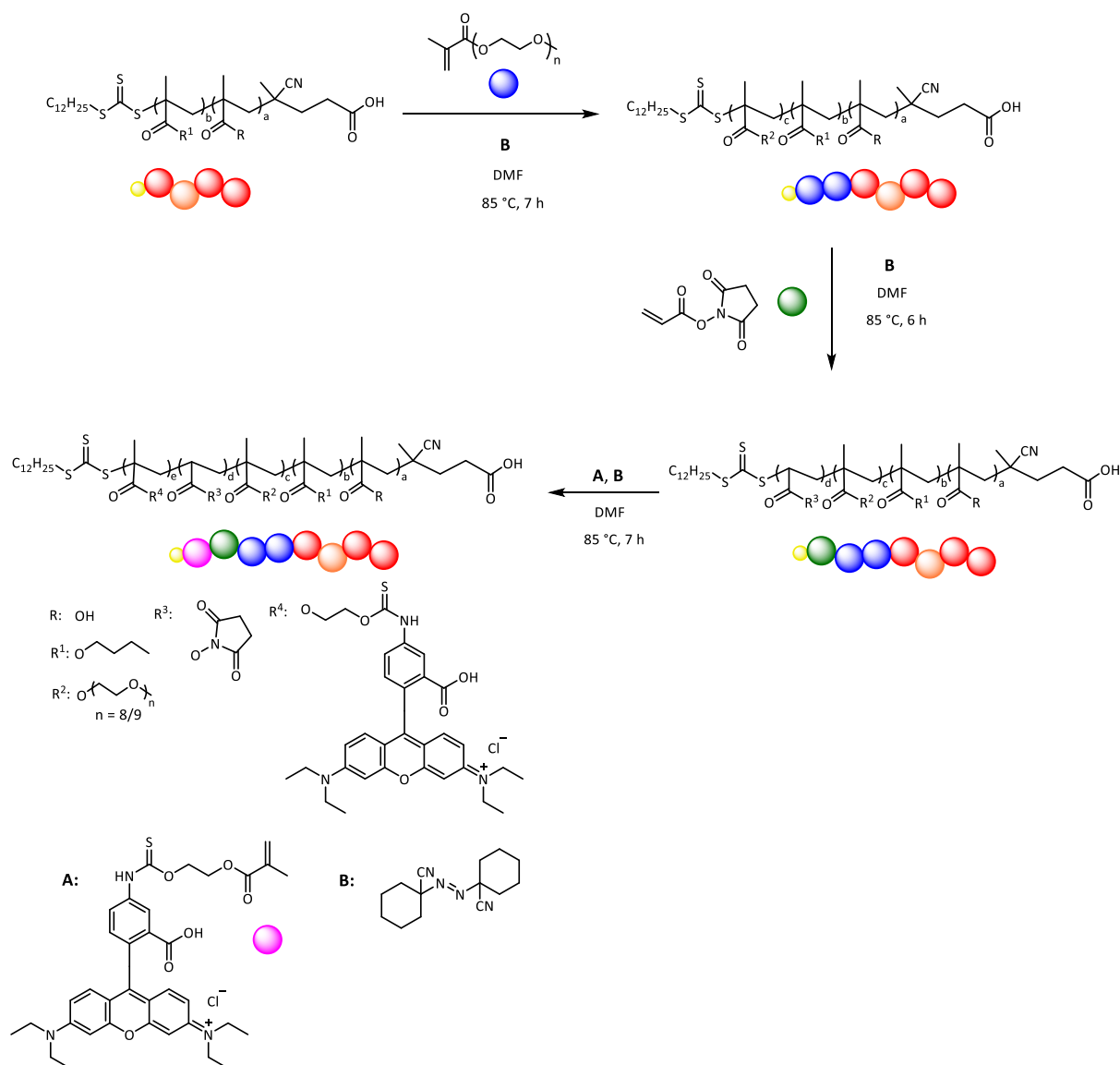
A second copolymer block (PEGMA-co-NAS) was added to the first BMA-co-MAA block. The MRhB fluorescent monomer was then incorporated in a separate polymerisation reaction to give BMA-co-MAA-*b*-PEGMA-co-NAS-*b*-MRhB.



Scheme 17. Block copolymerisation of BMA-co-MAA macroRAFT agent to give macroRAFT agent BMA-co-MAA-*b*-PEGMA. Followed by block copolymerisation of macroRAFT agent BMA-co-MAA-*b*-PEGMA to give rhodamine B labelled triblock copolymer. a = approx. 4, b = approx. 33, c = approx. 11, d = approx. 3, e = approx. 0.05.

Method D

A three step synthesis from the BMA-co-MAA macroRAFT agent to a rhodamine B labelled tetrablock copolymer: BMA-co-MAA-*b*-PEGMA-*b*-NAS-*b*-MRhB. The first block copolymerisation to add a PEGMA block was followed by addition of a NAS block and finally a MRhB block polymerisation.



Scheme 18. Block copolymerisation of BMA-co-MAA macroRAFT agent to give macroRAFT agent BMA-co-MAA-*b*-PEGMA. Followed by the addition of a NAS block and finally the incorporation of MRhB to give rhodamine B labelled tetrablock copolymer. a = approx. 4, b = approx. 33, c = approx. 11, d = approx. 3, e = approx. 0.05.

It was determined that larger micelles formed when a PEGMA-co-NAS block was synthesised (as in methods A and B), as opposed to two separate PEGMA-*b*-NAS blocks (as in methods C and D), results are displayed in **Table 32**. The size difference is potentially due to differences in PEGMA chain packing caused by the incorporation of another monomer into the block.²³⁵

The most direct synthesis route was to prepare the diblock copolymer detailed in method A. However, a library of BMA-co-MAA-*b*-PEGMA block copolymers had been previously synthesised. For this reason, step 2 of method C was used to chain extend the library of block

copolymer, incorporating both a crosslinkable-NAS unit and a fluorescent tag in preparation for biological uptake studies. Micelles were then crosslinked in water, see **Scheme 14**.

Method	Polymer description	Polymer M_n (Da)	Micelle diameter (nm)
A	1	9,100	29
B	2	8,200	38
C	3	8,200	17
D	4	7,700	18

Table 32. Comparison of micelles assembled from different polymer compositions (**Figure 40**) synthesised using methods A–D.

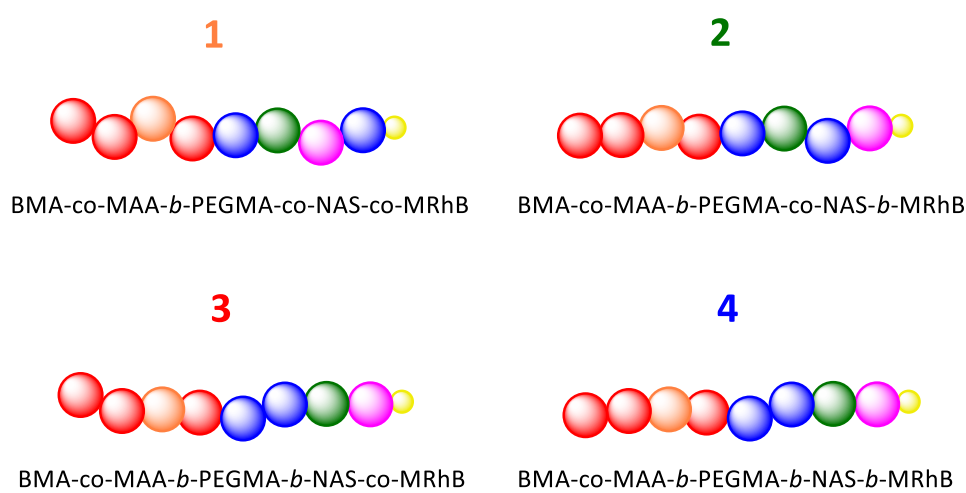


Figure 40. Different polymer block structures.

3.2.4 Fluorescent tagging and crosslinking of micelles for biological studies

Once crosslinking and fluorescent tagging methodology had been established synthesised BMA-co-MAA-*b*-PEGMA block copolymers, which formed a range of different size micelles, were fluorescently labelled and crosslinked in preparation for biological uptake studies. The number molecular weight (M_n) and dispersity (D_M) of polymers and the diameter of the crosslinked and labelled micelle particles are shown in **Table 33**. Fluorescently labelled and crosslinked micelles ranged in size from 14–170 nm. The cellular uptake of these micelles is discussed in **Chapter IV**.

Micelle	Synthesis	M_n (Da)	$\bar{D}_M$	Micelle diameter (nm)
(1)	QB	7,400	1.21	19 ±12
(2)	QB	8,200	1.18	18 ±6
(3)	QB	7,700	1.16	18 ±2
(4)	QB	8,000	1.24	24 ±2
(5)	PB	6,300	1.17	25 ±11
(6)	PB	3,800	1.18	25 ±11
(7)	PB	6,500	1.19	28 ±3
(8)	QB	8,900	1.29	36 ±13
(9)	PB	7,900	1.16	54 ±5
(10)	QB	10,400	1.33	42 ±16
(11)	PB	7,200	1.15	80 ±30
(12)	QB	7,700	1.34	46 ±20
(13)	PB	4,700	1.15	48 ±13
(14)	QB	10,000	1.31	83 ±50
(15)	QB	9,200	1.31	50 ±10
(16)	QB	12,000	1.42	54 ±28
(17)	QB	8,700	1.17	53 ±20
(18)	QB	11,500	1.38	73 ±40
(19)	QB	13,000	1.38	66 ±11
(20)	QB	12,000	1.41	53 ±29
(21)	QB	12,800	1.36	35 ±16
(22)	QB	8,200	1.37	63 ±7
(23)	QB	26,500	1.13	71 ±40
(24)	QB	9,100	1.31	79 ±20
(25)	QB	9,300	1.26	51 ±26
(26)	QB	10,300	1.24	44 ±19
(27)	QB	17,500	1.20	170 ±62

Table 33. M_n and $\bar{D}_M$ values for NAS-extended and rhodamine B tagged polymers for use in cellular uptake studies. Size of crosslinked micelles shown in nm. M_n and $\bar{D}_M$ values were calculated using GPC (THF SEC, Waters Empower-3 software).

Determining micelle fluorescence

As micelle uptake was quantified in terms of fluorescence in biological studies it was important that fluorescence was a true representation of uptake. For this reason, the molar ratio of rhodamine monomer: polymer chain was kept consistent in all reactions. Although the amount of rhodamine incorporated into the hydrophilic portion of the polymer chains should be consistent between samples, the fluorescence intensity of each sample was calculated using a concentration curve. This meant that micelle uptake could be normalised in terms of rhodamine fluorescence. This normalisation ensures that any detected uptake was a valid result.

Sample concentration versus fluorescence was plotted and the fluorescence intensity of each sample was calculated using the trend line equation obtained from the graph (**Figure 41**). The relative fluorescence unit (RFU) value was calculated at 250 µg/ml for each sample as this value lies in the middle of the range of concentrations tested. **Figure 41** is the plot of sample concentration versus relative fluorescence for micelle (**2**), the plots for all other samples can be found in the **Appendices**.

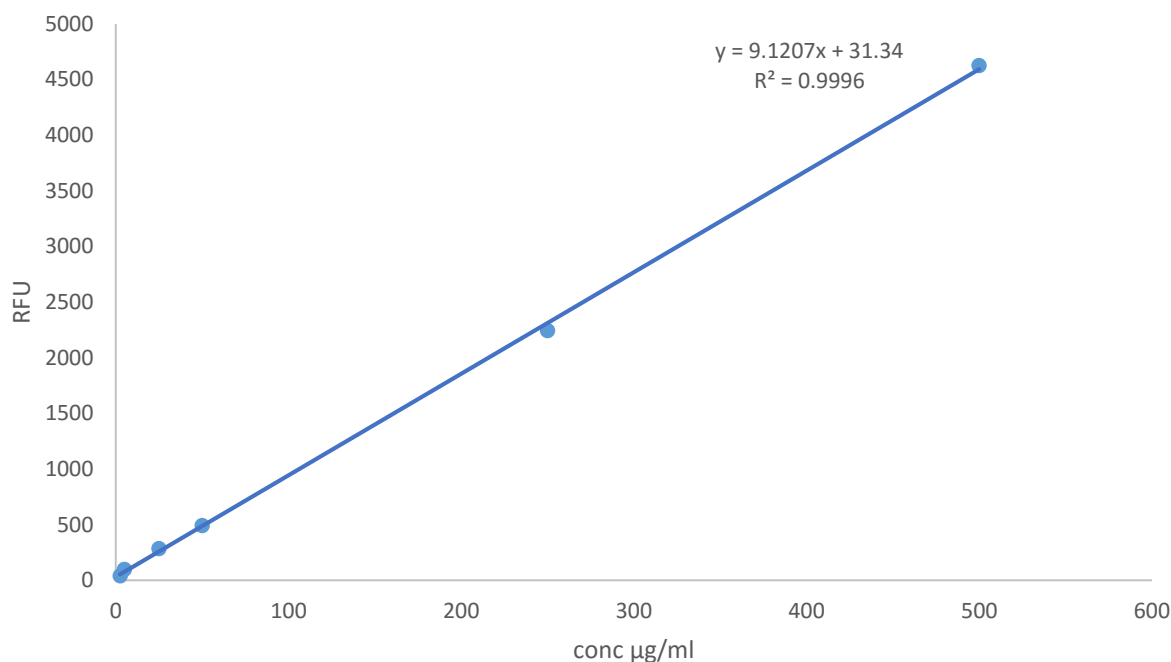


Figure 41. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle (**2**). The equation derived from the line of best fit ($y = 9.1207x + 31.34$) was used to calculate the RFU value for a 250 µg/ml solution.

3.3 Conclusions

The aim of the work described in this chapter was to prepare the library of selected micelles synthesised in **Chapter II** for cellular uptake studies (described in **Chapter IV**). To increase both their thermodynamic and kinetic stability, micelles were crosslinked using a diamine linker and succinimide units which were incorporated into the hydrophilic section of the polymer.²⁰⁹ This crosslinking system has the potential to be reversible in the presence of peptidases found inside macrophages as peptidases can cleave amide bonds.^{224–226} A rhodamine B fluorescent tag was also incorporated into the hydrophilic section of the polymer to allow for micelles to be tracked *in vitro*. Different methods to incorporate both the NAS and the rhodamine B monomers were investigated. The different methods involved incorporating the monomers at different points in the synthesis to give polymers with varying structures. To make the process as efficient as possible it was decided that the library of BMA-co-MAA-*b*-PEGMA block copolymers would be chain extended in one step to give BMA-co-MAA-*b*-PEGMA-*b*-NAS-co-RhB block copolymers. Once chain extended, all polymers were self-assembled in water and crosslinked. The success of the crosslinking step was confirmed by the stability of crosslinked micelles in THF and isopropanol, solvents which un-crosslinked micelles did not form in. After fluorescent tagging and crosslinking micelles were ready to be tested in *in vitro* cellular uptake studies.

3.4 Experimental

3.4.1 Materials and methods

Reagents and solvents were supplied from commercial sources and used as delivered unless otherwise described. Commercial suppliers used were: Sigma Aldrich, Thermofisher Scientific and VWR. The polyamine, JEFFAMINE D230 was kindly donated by HUNTSMAN, an American chemical manufacturer.²³⁶

The majority of GPC analysis was performed at CSIRO, Clayton, Australia on a Waters Alliance system equipped with an Alliance 2695 Separations Module (integrated quaternary solvent delivery, solvent degasser and autosampler system), a Waters column heater module, a Waters 2414 RDI refractive index detector, a Waters PDA 2996 photodiode array detector (210 to 400 nm at 1.2 nm) and 4 × Agilent PL-Gel columns (3 × PL-Gel Mixed C (5 μm) and 1 × PL-Gel Mixed E (3 μm) columns), each 300 mm × 7.8 mm², providing an effective molar mass range of 200 to 2 × 10⁶. Tetrahydrofuran (THF) high purity solvent (HPLC grade) was pre-filtered through aluminium oxide (90 active neutral, 70–230 mesh) with 0.45 μm filter, and 0.1 g/l 2,6-di-tert-butyl-4-methylphenol (BHT) was added as inhibitor. The filtered THF containing BHT was purged slowly with nitrogen gas and used as an eluent with a flow rate of 1 ml/min at 30 °C. Number (M_n) and weight average (M_w) molar masses were evaluated using Waters Empower-3 software. The GPC columns were calibrated with low dispersity polystyrene (PSt) standards (Polymer Laboratories) ranging from 580 to 7,500,000 g/mol, and molar masses are reported as PSt equivalents. A 3rd-order polynomial was used to fit the log M_p vs. time calibration curve, which was near linear across the molar mass ranges. GPC analysis was performed at CSIRO, Clayton, Australia.

Where specified GPC analysis was performed at Imperial College London using Agilent PL GPC-50 instrument, with SEC grade THF as the eluent at a flow rate of 1.0 mL/min at 40 °C. Two Polymer labs Mixed D columns were used in series. Polymers were dissolved in SEC grade THF and filtered prior to analysis. Number (M_n) and weight average (M_w) molar masses were evaluated using Agilent Cirrus GPC software. The GPC columns were calibrated with low dispersity polystyrene (PSt) standards (Polymer Laboratories) ranging from 570 to 3,600,000 g/mol, and molar masses are reported as PSt equivalents. A 3rd-order polynomial was used

to fit the $\log M_p$ vs. time calibration curve, which was near linear across the molar mass ranges.

A *N,N*-dimethylacetamide (DMAc) GPC system was also used where specified: Shimadzu system equipped with a CMB-20A controller system, a SIL-20AHT autosampler, a LC-20AT tandem pump system, a DGU-20A degasser unit, a CTO-20AC column oven, a RDI-10A refractive index detector and 4 x Waters Styragel columns (HT2, HT3, HT4, HT5 each 300 mm x 7.8 mm, providing an effective molar mass range of $100\text{--}4 \times 10^6$). This SEC system used DMAc (with 2.1 g/l of lithium chloride (LiCl)) as elutant with a flow rate of 1ml/min at 80 °C. The molar mass of the samples was obtained from a calibration curve constructed with poly(methyl methacrylate) (PMMA) standards (Polymer Laboratories) of low dispersity ($\mathcal{D}$) value. A 3rd order polynomial was used to fit the $\log M_p$ vs. time calibration curve, which was linear across the molar mass ranges.

DLS measurements were performed on a Beckman Coulter DelsaNano C Particle Analyzer. BRAND 1.5 ml semi-micro polystyrene (PSt) cuvettes were used. *Measurement parameters*: repetition: 5, temperature: 25 °C, equilibration: 20 sec, wait time: 120 sec, dust limit: 10, upper dust limit: 25, lower dust limit: 50, optimum intensity: 20,000, maximum intensity: 30,000, minimum intensity: 10,000, pinhole: 50 μm . *Analysis parameters*: analysis method: CONTIN, weight function: 1, fitting range: $G_2(\tau)$, $G_2(\tau)_{\text{max}}$: 2, $G_2(\tau)_{\text{min}}$: 1.003. *Cell parameters*: measurement item: size, cell type: size cell, cell centre Z: 4.85 mm, cell centre X: 6.55 mm, correlator type: log, accumulation times: 100. *Dilutant parameters*: dilutant name: water, refractive index: 1.33, viscosity: 0.89, dielectric constant: 78.3. DLS measurements were performed at Imperial College London.

^1H NMR spectra were recorded on a Bruker DRX-400 spectrometer operating at 400 MHz. Chemical shifts (δ) are quoted in units of parts per million (ppm) downfield from tetramethylsilane and are referenced to a residual solvent peak (CDCl_3 (δ_{H} : 7.26)). Coupling constants (J) are quoted in units of Hertz (Hz). The following abbreviations are used for ^1H NMR analysis: s = singlet, br s = broad singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet. Spectra were obtained using the Imperial College London Department of Chemistry NMR Service.

Unless stated otherwise polymers were purified *via* dialysis. Benzoylated dialysis tubing, with an average flat width of 9 mm, was used. Tubing capacity approximately 10 mL/ft, pore size: 2000 MWCO, purchased from Sigma Aldrich. Reaction mixture containing synthesised polymer was pipetted into the dialysis tubing and dialysis tubing closures (Sigma Aldrich) were placed at each end. Dialysis tubing containing the solution was then placed in 0.5–1.0 l deionised water for 48–72 h. Water was replaced every 24 h.

3.4.2 Determination of micelle stability

Effect of concentration on micelle stability

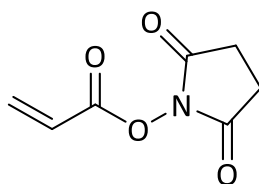
Solutions of different concentrations of block copolymer BMA-co-MAA-*b*-PEGMA, dissolved in water, were made up and analysed *via* the pendant drop method and DLS to determine the CMC. The concentrations used for the Pendant drop method were: 1.0×10^{-4} , 7.5×10^{-5} , 5.0×10^{-5} , 2.5×10^{-5} , 1.3×10^{-5} , 6.3×10^{-6} , 3.1×10^{-6} , 1.6×10^{-6} and 7.8×10^{-7} mol/dm³. The concentrations used for DLS analysis were: 5.0×10^{-5} , 2.5×10^{-5} , 1.3×10^{-5} , 6.3×10^{-6} , 3.1×10^{-6} , 1.6×10^{-6} and 7.8×10^{-7} mol/dm³. DLS analysis was repeated with block copolymer BMA-co-MAA-*b*-PEGMA-co-NAS to ensure that the addition of the NAS monomer to the polymer did not affect micelle formation.

Effect of heat on micelle stability

0.5 mg/ml solutions of block copolymer BMA-co-MAA-*b*-PEGMA-co-NAS in water were made and DLS analysis was carried out at 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 °C. It was not possible to heat the solutions above 70 °C using the DLS equipment.

3.4.3 Crosslinking methodology

Synthesis of N-Acryloxysuccinimide

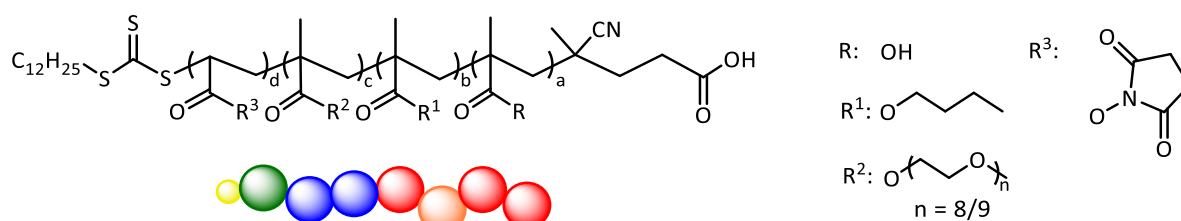


N-hydroxysuccinimide (NHS) (11.5 g, 100 mmol) and triethylamine (15 ml, 110 mmol) were dissolved in CHCl₃ (15 ml) at 0 °C to give a clear solution. Acryloyl chloride (10 g, 110 mmol)

Ratio PEGMA: NAS	PEGMA	NAS
10: 3	0.63 ml, 1.43 mmol	72 mg, 0.43 mmol
2: 1	0.59 ml, 1.34 mmol	113 mg, 0.67 mmol

Table 34. Quantities of monomers PEGMA and NAS used in quasi-diblock and quasi-triblock copolymer synthesis. The PEGMA: NAS ratio was varied in both diblock and triblock synthesis to monitor the dependence of successful crosslinking on the amount of NAS in the polymer chain.

Synthesis of BMA-co-MAA-*b*-PEGMA-*b*-NAS triblock copolymer



a = approx. 4, b = approx. 33, c = approx. 11, d = approx. 3.

Reagents BMA (0.72 ml, 4.5 mmol), MAA (0.40 ml, 0.50 mmol), RAFT agent 4-cyano-4[(dodecylsulfenylthio carbonyl)sulfanyl] pentanoic acid (60 mg, 0.15 mmol), ACHN (3.6 mg, 0.015 mmol) and trioxane (approx. 15 mg) were dissolved in DMF (2.5 ml) and added to a Young's tap Schlenk flask. The solution was degassed *via* the freeze-pump-thaw method (3 x 20 min cycles) and then heated at 85 °C for 9 h. Upon cooling, NAS and ACHN (see **Table 35** for quantities) and DMF (0.5 ml) were added to the Schlenk flask. As very small quantities of ACHN were required, larger quantities were weighed out and diluted. The reaction mixture was then degassed *via* the freeze-pump-thaw method (3 x 20 min cycles) before it was heated at 85 °C for 6 h.

Ratio of PEGMA: NAS	NAS	ACHN
10: 3	73 mg, 0.43 mmol	0.3 mg, 1 µmol
2: 1	110 mg, 0.67 mmol	0.4 mg, 2 µmol

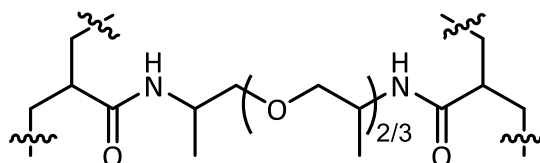
Table 35. Quantities of NAS and ACHN used in step 2 of triblock copolymer synthesis.

Once the reaction had cooled to room temperature PEGMA, ACHN (see **Table 36** for quantities) and DMF (0.5 ml) were added to the reaction vessel. The reaction mixture was then degassed *via* the freeze-pump-thaw method (3 x 20 min cycles) before it was heated for a further 7 h at 85 °C. The quasi-triblock copolymer was purified *via* dialysis followed by lyophilisation to give a yellow gum. ¹H NMR analysis showed no traces of solvent or monomers in the product. *M_n*: 8,100, *Đ_M*: 1.16.

Ratio of PEGMA: NAS	PEGMA	ACHN
10: 3	680 mg, 1.4 mmol	1 mg, 4 μ mol
2: 1	640 mg, 1.3 mmol	1 mg, 4 μ mol

Table 36. Quantities of PEGMA and ACHN used in step 3 of triblock copolymer synthesis.

Crosslinking reaction



Polymer sample (either quasi-diblock or quasi-triblock copolymer) was dissolved in deionised water (250 ml) and the polyetheramine JEFFAMINE D230 was added under N₂ (see **Table 37** for quantities). The reaction was stirred at room temperature for 20 h. Solvent was removed under reduced pressure.

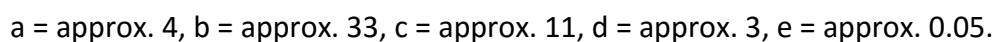
Polymer	Polymer	JEFFAMINE
BMA-co-MAA-b-NAS-b-PEGMA 2:1 PEGMA: NAS ratio	19 mg, 2.0 μ mol	1 mg, 5 μ mol
BMA-co-MAA-b-PEGMA-co-NAS 10:3 PEGMA: NAS ratio	32 mg, 4.0 μ mol	2 mg, 10 μ mol
BMA-co-MAA-b-NAS-b-PEGMA 10:3 PEGMA: NAS ratio	35 mg, 4.0 μ mol	2 mg, 10 μ mol

Table 37. Polymer and diamine quantities used in crosslinking reactions.

Analysis of crosslinked micelles

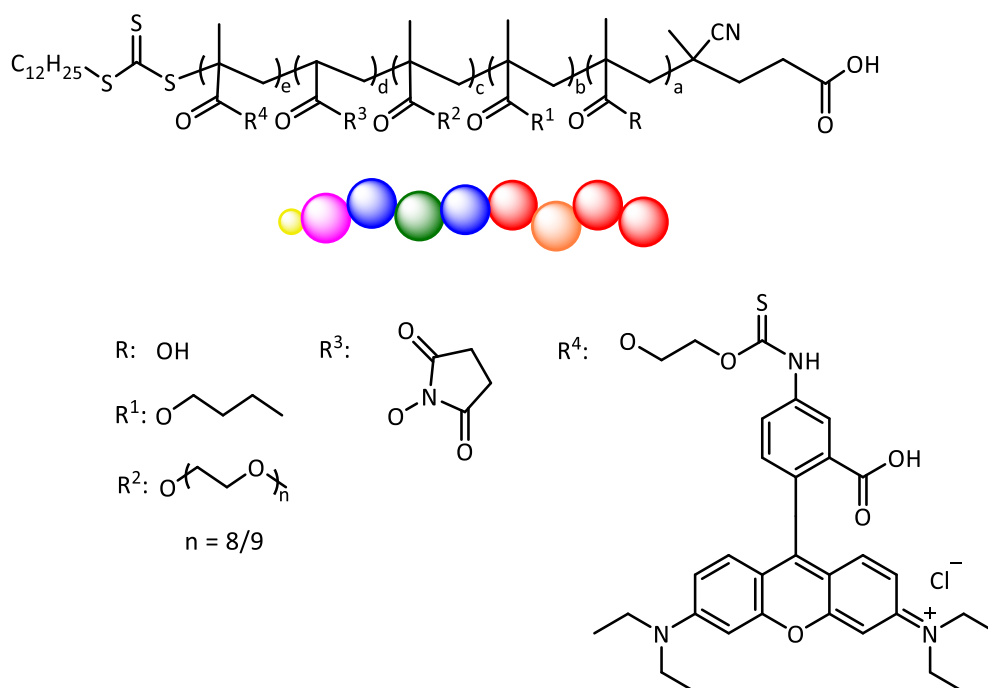
Polymer samples were made up to a concentration of 0.5 mg/ml in all solvents. Solvents used in study: deionised water, hexane, THF, isopropanol and toluene. A 1 ml quartz cuvette was used in place of a polystyrene cuvette in all crosslinking studies due to the solubility of polystyrene in some solvents.

Synthesis of fluorescent diblock copolymer BMA-co-MAA-*b*-PEGMA-co-NAS-co-MRhB



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Synthesis of fluorescent triblock copolymer BMA-co-MAA-*b*-PEGMA-co-NAS-*b*-MRhB



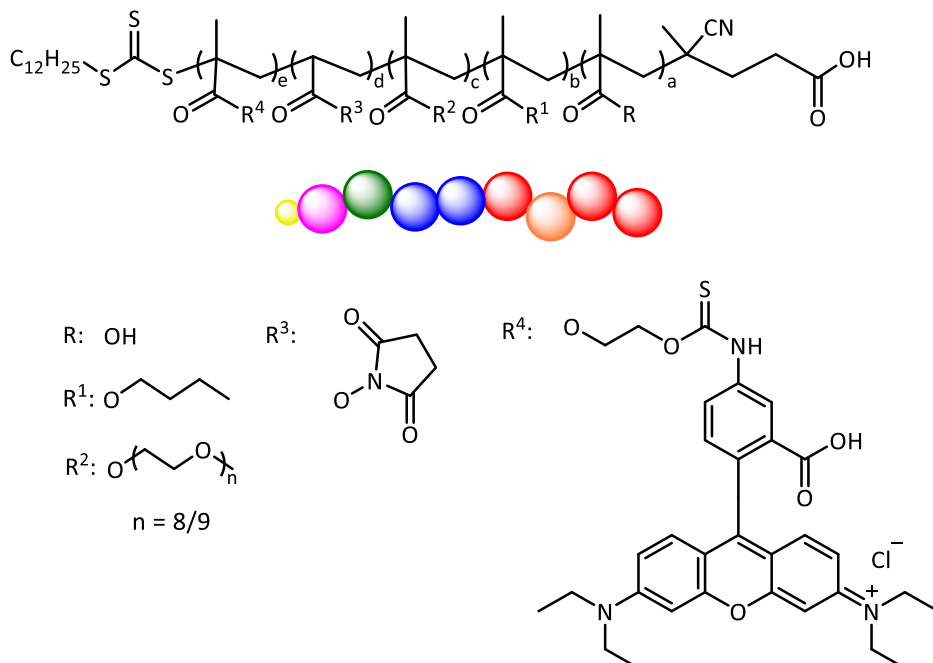
a = approx. 4, b = approx. 33, c = approx. 11, d = approx. 3, e = approx. 0.05.

BMA (0.72 ml, 4.5 mmol), MAA (0.4 ml, 0.5 mmol), ACHN (4 mg, 0.02 mmol), RAFT agent 4-cyano-4[(dodecylsulfenylthio carbonyl)sulfanyl] pentanoic acid (60 mg, 0.15 mmol) and trioxane (approximately 10 mg) were dissolved in DMF (2.5 ml). The solution was then transferred to a Young's tap Schlenk flask and the mixture was degassed *via* the freeze-pump-thaw method (3 x 20 min cycles). After the solution had thawed to room temperature it was heated at 85 °C for 9 h. Upon completion of the reaction the mixture was allowed to cool before PEGMA (0.69 ml, 1.57 mmol), ACHN (2 mg, 7 μmol) and DMF (1 ml) were added to the reaction vessel. The mixture was then degassed again, *via* the freeze-pump-thaw method (3 x 20 min cycles) and then allowed to thaw to room temperature before being heated at 85 °C for 7 h. Upon cooling the quasi-block copolymer was purified *via* dialysis and isolated using lyophilisation.

MacroRAFT agent (160 mg, 0.02 mmol), NAS (10 mg, 0.06 mmol), methacryloxyethyl thiocarbamoyl rhodamine B (1 mg, 1 μmol) and ACHN (0.5 mg, 2 μmol) were dissolved in DMF (1.5 ml). Due to the small quantity of ACHN required a larger amount was weighed out and diluted. The solution was transferred to a Young's tap Schlenk flask before being degassed *via* the freeze-pump-thaw method (3 x 20 min cycles). The degassed solution was heated at 85 °C

for 7 h. Once cooled, the triblock copolymer was purified *via* dialysis and water was removed using lyophilisation. M_n : 8,200, $\bar{D}_M$: 1.37.

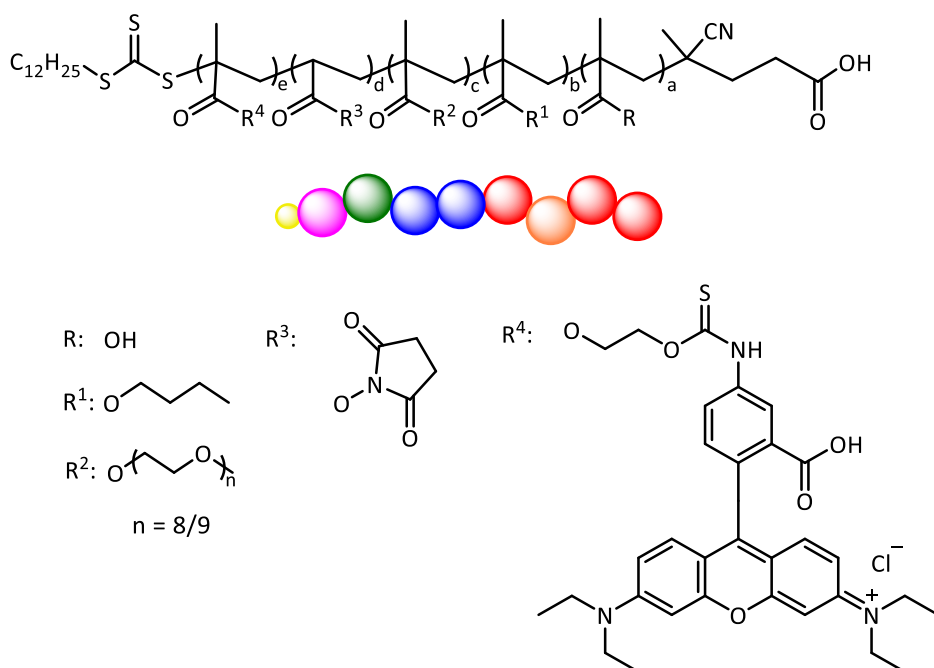
Synthesis of fluorescent triblock copolymer BMA-co-MAA-*b*-PEGMA-*b*-NAS-co-MRhB



a = approx. 4, b = approx. 33, c = approx. 11, d = approx. 3, e = approx. 0.05.

Diblock copolymer BMA-co-MAA-*b*-PEGMA was synthesized as described in 'Synthesis of fluorescent triblock copolymer BMA-co-MAA-*b*-PEGMA-co-NAS-*b*-MRhB'. The macroRAFT agent, BMA-co-MAA-*b*-PEGMA, (160 mg, 20 μmol), NAS (10 mg, 60 μmol), MRhB (1 mg, 1 μmol) and ACHN (0.5 mg, 2 μmol) were dissolved in DMF (1.5 ml). Due to the small quantity of ACHN required, a larger amount was weighed out and diluted. The reaction mixture was transferred to a Young's tap Schlenk flask and then degassed using the freeze-pump-thaw method (3 x 20 min cycles) before being heated at 85 $^{\circ}\text{C}$ for 7 h. The product was purified *via* dialysis in water and then lyophilised to give a pink gum. M_n : 8,200, $\bar{D}_M$: 1.18.

Synthesis of fluorescent tetrablock copolymer BMA-co-MAA-*b*-PEGMA-*b*-NAS-*b*-MRhB



a = approx. 4, b = approx. 33, c = approx. 11, d = approx. 3, e = approx. 0.05.

BMA, MAA, ACHN (3.6 mg, 0.015 mmol), RAFT agent 4-cyano-4[(dodecylsulfenylthio carbonyl)sulfanyl] pentanoic acid (60 mg, 0.15 mmol) and trioxane (approximately 10 mg) were dissolved in DMF (2.5 ml). The quantities of monomers added can be found in **Table 38**. The solution was then transferred to a Young's tap Schlenk flask and the mixture was degassed *via* the freeze-pump-thaw method (3 x 20 min cycles). After the solution had thawed and warmed to room temperature it was heated at 85 °C for 9 h. Upon completion of the reaction the mixture was allowed to cool before PEGMA (0.69 ml, 1.6 mmol), ACHN (2 mg, 7 μmol) and DMF (1 ml) were added to the reaction vessel. The mixture was then degassed again, *via* the freeze-pump-thaw method (3 x 20 min cycles) and then allowed to thaw to room temperature before being heated at 85 °C for 7 h. Upon cooling the quasi-block copolymer was purified *via* dialysis.

MacroRAFT agent (160 mg, 0.02 mmol), NAS (10mg, 0.06 mmol) and ACHN (0.5 mg, 2 μmol) were dissolved in DMF (1.5 ml). Due to the small quantity of ACHN required, a larger amount was weighed out and diluted. The solution was transferred to a Young's tap Schlenk flask and degassed *via* the freeze-pump-thaw method (3 x 20 min cycles) before being heated at 85 °C for 6 h. Upon cooling, MRhB (1 mg, 1 μmol) was added (dissolved in minimal DMF) and the

solution was degassed again *via* the freeze-pump-thaw method (3 x 20 min cycles). The degassed solution was heated at 85 °C for 7 h. Once cooled the tetrablock copolymer was purified *via* dialysis and isolated using lyophilisation. M_n : 7,700, $\bar{D}_M$: 1.16.

3.4.5 Fluorescently labelling and crosslinking polymer library

Polymer, NAS, MRhB and ACHN (see **Table 38** for quantities) were dissolved in DMF (1.5 ml). Due to the small quantity of ACHN required, larger amounts were weighed out and diluted. The reaction mixtures were transferred to a Young's tap Schlenck flask and then degassed using the freeze-pump-thaw method (3 x 20 min cycles) before being heated at 85 °C for 7 h. The products were purified *via* dialysis in water and then lyophilised to isolate the product.

To crosslink polymers (7 μ mol) were dissolved in H₂O (500 ml). The polyetheramine, JEFFAMINE D230 was added (2 mg, 10 μ mol) under nitrogen and the reaction was stirred for 20 h at room temperature. Due to solubility issues methanol had to be added to some reaction mixtures prior to the addition of JEFFAMINE. In such instances methanol was removed once the polymer had dissolved and did not affect crosslinking.

Sample	Polymer	NAS	MRhB	ACHN
(1)	160 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(2)	160 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(3)	160 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(4)	160 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(5)	120 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(6)	81 mg, 14 μ mol	7 mg, 40 μ mol	1 mg, 1 μ mol	0.3 mg, 1 μ mol
(7)	130 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(8)	170 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(9)	140 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(10)	250 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(11)	62 mg, 10 μ mol	5 mg, 30 μ mol	0.5 mg, 0.5 μ mol	0.3 mg, 1 μ mol
(12)	150 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(13)	100 mg, 16 μ mol	8 mg, 40 μ mol	1 mg, 1 μ mol	0.4 mg, 2 μ mol
(14)	180 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(15)	180 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(16)	310 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(17)	160 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(18)	280 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(19)	320 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(20)	250 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(21)	320 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(23)	260 mg, 10 μ mol	5 mg, 30 μ mol	0.5 mg, 0.5 μ mol	0.3 mg, 1 μ mol
(25)	230 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol
(26)	350 mg, 30 μ mol	14 mg, 80 μ mol	1 mg, 1 μ mol	0.7 mg, 3 μ mol
(27)	330 mg, 20 μ mol	10 mg, 60 μ mol	1 mg, 1 μ mol	0.5 mg, 2 μ mol

Table 38. Quantities used in polymerisation reactions to incorporate NAS and MRhB in library of block copolymers.

Determining micelle fluorescence

All fluorescence measurements were performed using an EnSpire Multimode Plate Reader (PerkinElmer). Samples were loaded in 96 Nunc DELFIA Strip plates.

Initial fluorescence intensity scans were performed to confirm the maximum emission and excitation values for rhodamine labelled micelles. To determine maximum emission a scan was run from 565–705 nm using an excitation wavelength of 550 nm. To determine maximum excitation a scan was run from 400–600 nm, using an emission wavelength of 620 nm. At an excitation wavelength of 550 nm, Em_{max} : 575 nm. At an emission wavelength of 620 nm, Ex_{max} : 555 nm. An emission scan was run from 570–670 nm, using 555 nm as the excitation wavelength. An excitation scan was run from 400–560 nm using 575 nm as the emission wavelength. The emission maximum was determined to be 575 nm and excitation maximum 555 nm.

A series of solutions were made up in phosphate buffer solution (PBS) MW: 310 for each sample at the following concentrations: 500, 250, 50, 25, 5 and 2.5 $\mu\text{g/ml}$, lower concentrations were made up as dilutions of the higher concentrations to help ensure consistency. 200 μl of each solution was made up, with 50 μl was used per measurement. $N = 3$ measurements were taken at each concentration. Blanks of an empty well and of the phosphate buffer solution were run, relative fluorescence of blanks was < 60 RFU. Rhodamine fluorescence was measured using an excitation wavelength of 555 nm and an emission wavelength of 575 nm (determined by the fluorescence intensity scan).

Chapter IV

Cellular Uptake Studies

4.1 Introduction

4.1.1 Cellular studies

In vitro studies to test drug delivery systems give an indication of how the delivery system will work *in vivo*. Mice are commonly used in *in vitro* and *in vivo* studies to test drugs and drug delivery systems.²³⁷ The genetic make-up of a mouse is similar to that of humans and they contract many of the same diseases; for these reasons mice are good models for potential human treatments.²³⁸ In this project bone marrow and peritoneal cells were harvested from mice to analyse the cellular uptake of synthesised polymeric micelles. The uptake of micelles by macrophage and non-macrophage populations were compared as macrophages were the delivery target in this work (see section 1.2.1).

Bone marrow

In mice, bone marrow is located inside the medullary cavity of axial and long bones. These include the skull, vertebrae, ribs, shoulders, pelvis, legs, arms, hands and feet.²³⁹ Bone marrow is important as it provides structural support in the body and because it is where haematopoiesis, the formation of blood cells, occurs. Blood cells are synthesised in the bone marrow and then released into the blood stream. Approximately six billion blood cells per kilogram of total body weight are thought to be produced by the bone marrow per day.²³⁹ Haematopoiesis needs an extensive and structured support system so that essential factors such as cytokines can be transported around the tissue and so formed blood cells can leave and enter the blood stream. Evidence suggests that the cytokines and other factors required for haematopoiesis are synthesised locally in the bone marrow.^{240–244}

Cytokines play a role in the differentiation of stem cells into various different cell types. Erythrocytes, granulocytes, monocytes and lymphocytes are all formed in and released from the bone marrow (**Figure 42**).²⁴⁵

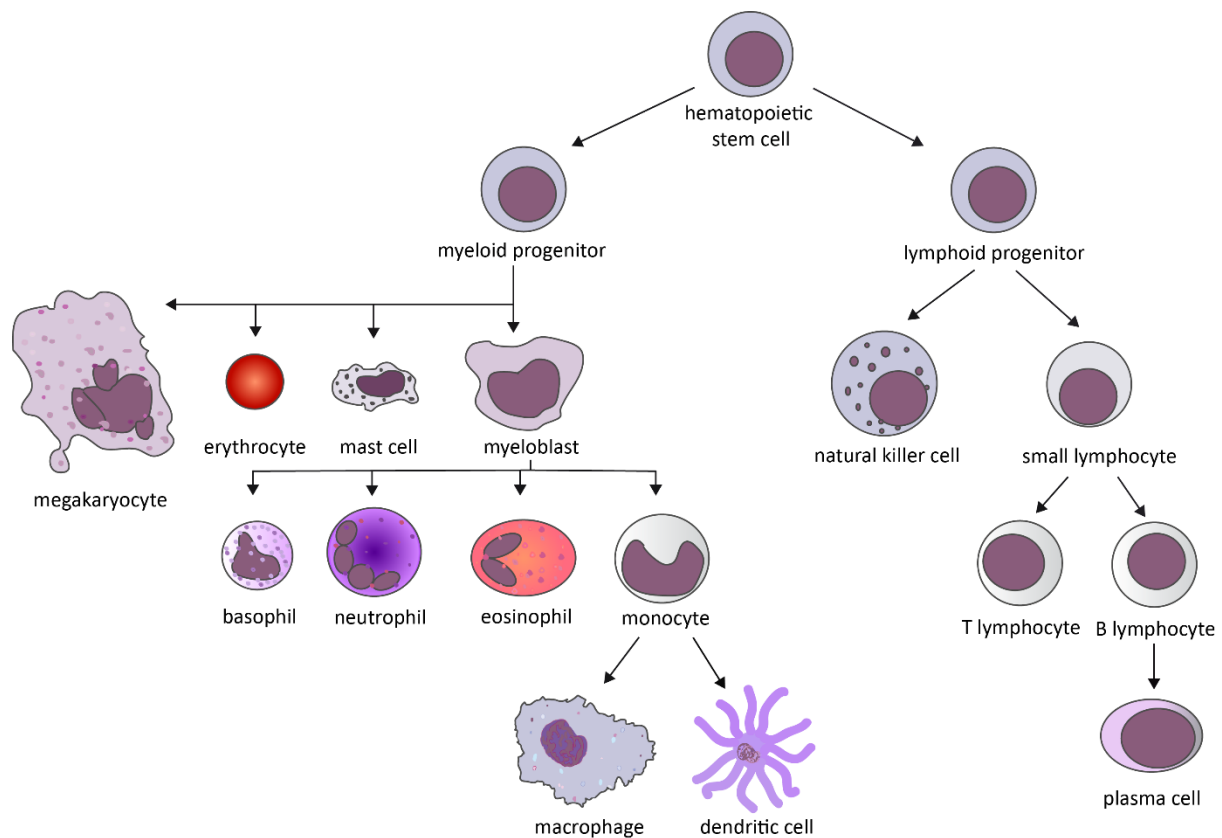


Figure 42. Differentiation of hematopoietic stem cells in the bone marrow.

The haematopoietic tissue in bone marrow is made up of many different cell types at different stages of haematopoietic development, including myeloblasts, erythroblasts and haematopoietic stem cells.^{245,246} As haematopoietic cells develop, their morphology becomes more distinct, **Figure 42** shows the main lineages of haematopoiesis: megakaryocytes basophils, neutrophils, eosinophils, monocytes, and T and B lymphocytes.²⁴⁶ As well as cells that play an active role in haematopoiesis, the bone marrow also contains stromal cells which support haematopoiesis. Stromal cells include: mesenchymal stem cells, these differentiate into structures that form bone, cartilage and muscle; fibroblasts; adipocytes (fat cells); osteoblasts, which synthesise bone; endothelial cells and endothelial stem cells.^{245,247} Stromal cells are very important in the differentiation of monocytes to macrophages (discussed in detail in section 1.2) as they produce the growth factor CSF-1, previously discussed in section 1.2.1, which is involved in the formation and regulation of macrophages.⁴⁴

Macrophages are commonly harvested from the bone marrow for *in vitro* studies.²⁴⁸ Macrophages can also be harvested from the peritoneal cavity. Macrophages harvested from the peritoneal cavity are more mature than bone marrow macrophages, but are found in

lower abundance. A third option, not explored in this work, is to harvest macrophages from the spleen.²⁴⁸

Peritoneal cavity

The peritoneum is a membrane-enclosed cavity found in mammals. Organs inside this cavity include the liver, spleen and intestine. The organs are surrounded by a cellular fluid; Ito *et al.* studied free murine cells harvested from the peritoneum and determined that there were three different types of cells present: lymphocytes, mononuclear cells and macrophages.²⁴⁹

Macrophages found in the peritoneal cavity are primarily M1 macrophages; macrophages responsible for fighting invading pathogens or other materials harmful to the host (as opposed to M2 macrophages which work to fix damaged tissue in the host).^{250,43} Differences between M1 and M2 macrophages are further outlined in section **1.2**.

Cellular uptake mechanisms

One of the aims of this work was to selectively target macrophages *via* the mechanism of phagocytosis. As discussed in **Chapter I**, macrophages are the most well-known phagocytosing cell. Phagocytosis is a type of endocytosis, there are three other categories of endocytosis; pinocytosis, clathrin-mediated endocytosis (or receptor mediated endocytosis) and caveolae.²⁵¹ Phagocytosis involves the internalisation of much larger particles than other methods of endocytosis. Phagocytic cells, such as macrophages, can be targeted by designing particles which can be internalised *via* phagocytosis but are too large to be internalised *via* other mechanisms of endocytosis.^{96,97}

In phagocytosis, receptors on the surface of the cell bind to foreign material, the macrophage then ‘reaches around’ the foreign material and engulfs it, trapping it in a phagosome inside the cell.⁶⁵ Phagosomes combine with lysosomes or granules to form phagolysosomes and the foreign materials are destroyed inside phagolysosomes by enzymes and various different chemicals, including reactive oxygen and nitrogen species.²⁵² Macrophages release part of the undigested material to recruit more phagocytes to the site.²⁵³ Pinocytosis is best described as ‘cell drinking’, it is the uptake of fluids from the area surrounding the cell. Unlike other types of endocytosis, pinocytosis is nonspecific; any dissolved compound can be taken up *via* pinocytosis.⁶⁵ In clathrin-mediated endocytosis material is internalised *via* a clathrin-coated

pit which forms in the cell's plasma membrane and then forms a vesicle inside the cell.²⁵⁴ Once the vesicle has fully formed inside the cell it loses its clathrin coating and combines with an endosome where it is processed by the cell; material will either be recycled, transferred to a lysosome and broken down, or used by the cell.²⁵⁵ The mechanism of caveolae is similar to clathrin-mediated endocytosis but the plasma membrane pits are not clathrin coated, instead the pits are coated by caveolin, cholesterol and glycolipids.^{256,51}

4.1.2 Micelle encapsulation

There are a number of methods for encapsulating drugs (or other types of cargo) in a micelle particle. The best method to use depends on both the micelle and the cargo being encapsulated. As discussed in section **3.1.1**, micelle stability is crucial for targeted drug delivery. Micelles have to be stable enough to reach the target destination without disassembling and yet have to disassemble upon internalisation in the target cells to ensure effective drug release and clearance of the carrier from the body after delivery. Micelle stability is not the only issue that has to be considered in terms of premature drug release; the cargo has to be stably encapsulated within the micelle or leakage can occur as micelles travel to the target destination. Drug leakage can cause unwanted side effects and prevents the maximum amount of drug from being delivered to the target cells.

There are three main ways to incorporate a drug into a micelle delivery system. The drug can be combined with the polymer chains before assembly (these polymers are known as polymer-drug conjugates). Another option is for drugs to be encapsulated and held inside the core of the micelle *via* hydrophobic interactions, charge interactions or hydrogen bonding. The third method of incorporation involves polymer and drug moieties with different charges which are neutralised when self-assembly to form micelles occurs.⁷ If a drug is incorporated *via* hydrogen bonding, charge interactions or hydrophilic-hydrophobic interactions with the micelle then micelle disassembly is enough to release the drug, as the interactions are no longer present once the micelle has disassembled. However, if the drug is conjugated to the micelle or to the polymers within the micelle, then the release of the drug has to be engineered *via* the cleavage of the bonds between the drug and the polymer. This introduces a higher level of complication within the delivery system.

There are a number of methods to encapsulate a drug in the core of micelle particles (**Figure 43**). If the drug is soluble, or sparingly soluble in water then the drug can be mixed with a solution of the micelles and will be incorporated into the core of the micelles *via* diffusion. The success of this method varies significantly between individual systems. Another method, referred to as the dialysis method involves block copolymers and the drug being dissolved in an organic solvent (in which micelles do not form) and then dialysed in water, resulting in the formation of micelle particles with incorporated drug molecules.²⁰⁵ The cosolvent evaporation method works in the same way as the dialysis method, the only difference is water is gradually added and then the solvent is removed *via* evaporation, as opposed to dialysis. In the emulsion method, polymer and drug are dissolved in an organic solvent which is then added to an aqueous phase and mixed at high speed to create an emulsion. The organic solvent is then removed to leave the drug encapsulated in polymer particles. In the solvent evaporation method, polymer and drug are dissolved in a solvent which is then removed, leaving a solid mixture of polymer and drug. When the solid is redissolved in water, micelles are formed with the drug encapsulated inside.

Goepferich *et al.* synthesised PEGylated poly-4-(vinylpyridine) micelles and compared different encapsulation methods for loading the steroid dexamethasone.¹⁶⁷ The encapsulation methods compared were dialysis, oil/water emulsion and cosolvent evaporation. The group found that dialysis achieved <2% w/w (weight per weight), the oil/water emulsion method achieved approximately 13% w/w loading and cosolvent evaporation resulted in approximately 19% w/w loading.¹⁶⁷

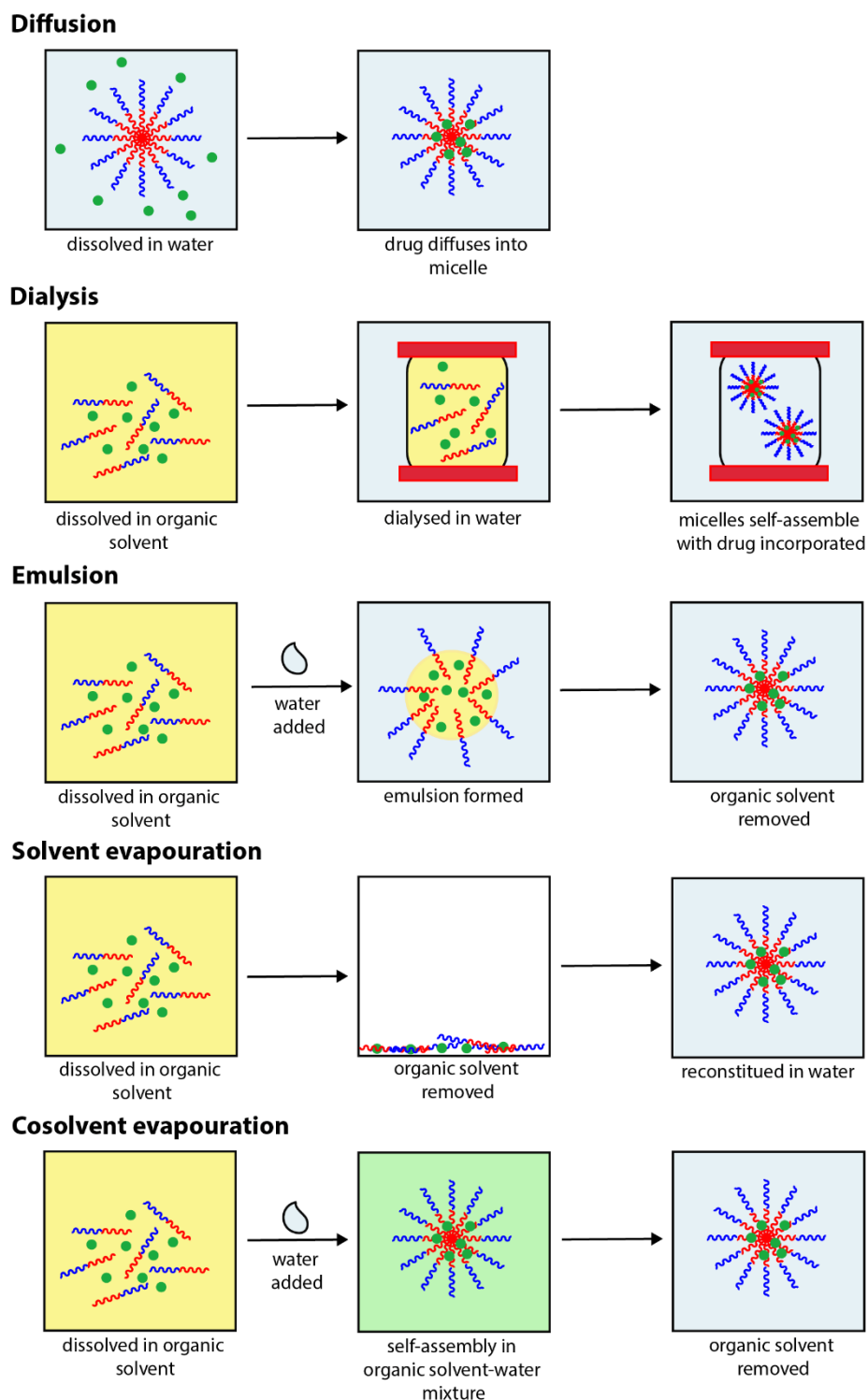


Figure 43. Schematic illustration of different encapsulation methods.

The cellular uptake of synthesised micelles by macrophages and non-macrophages is compared in this chapter. A library of micelles synthesised in **Chapter II**, then crosslinked and fluorescently labelled in **Chapter III** was tested in studies with macrophages and non-macrophages. The term ‘non-macrophages’ refers to cells harvested from the bone marrow

which are not macrophages, e.g. megakaryocytes, granulocytes, endothelial cells, fibroblasts, mesenchymal stem cells, adipocytes, osteoblasts, osteoclasts and platelets.^{245,247} Micelles were tested on macrophages harvested from both the bone marrow and the peritoneal cavity. It was hypothesised that the micelles would not be large enough to achieve selective phagocytosis by macrophages but that micelle size would have an effect on uptake. This hypothesis was based on the results from a number of studies (discussed in section **1.2.2**) which have determined that the size of a particle has an effect on its uptake by macrophages.^{39,63,96–98} In terms of the cut-off size for selective phagocytosis by macrophages different limits have been reported, but the general consensus is particles of at least 500 nm are required.^{63,96–98}

The encapsulation of the fluorescent dye, 4',6-diamidino-2-phenylindole (DAPI), in micelles *via* the diffusion method is also discussed in this chapter. It was hypothesised that favourable interactions between the acidic micelle core and basic dye would help stabilise the encapsulation of the cargo.

All animal work involved in this project was performed at CSIRO, in the Nilsson group. The Nilsson group's expertise in the areas of endocytosis and phagocytosis made their contribution to the project invaluable.

4.2 Results and Discussion

Cellular uptake studies were performed to determine whether synthesised micelle particles were phagocytosed by murine macrophages. 27 micelle particles of a range of different sizes and compositions were selected for these studies. Micelles were all fluorescently labelled with rhodamine B and crosslinked (**Chapter III**). Details of each of the selected particles can be found in **Table 39**.

Micelle	Synthesis	Polymer description	BMA: MAA ratio	Polymer MW (Da)	Micelle diameter (nm)
(1)	QB	A	9: 1	7,400	14
(2)	QB	A	9: 1	8,200	18
(3)	QB	B	9: 1	7,700	18
(4)	QB	E	9: 1	8,000	24
(5)	PB	A	2: 1	6,300	25
(6)	PB	A	2: 1	3,800	25
(7)	PB	A	4: 1	6,500	27
(8)	QB	A	9: 1	8,900	36
(9)	PB	A	9: 1	7,900	40
(10)	QB	A	9: 1	10,400	42
(11)	PB	A	9: 1	7,200	42
(12)	QB	A	2: 1	7,700	46
(13)	PB	A	4: 1	4,700	48
(14)	QB	A	9: 1	10,000	49
(15)	QB	A	9: 1	9,200	50
(16)	QB	A	9: 1	12,000	52
(17)	QB	A	4: 1	8,700	53
(18)	QB	A	9: 1	11,500	54
(19)	QB	A	9: 1	13,000	56
(20)	QB	A	9: 1	12,000	57
(21)	QB	A	9: 1	12,800	59
(22)	QB	C	9: 1	8,200	63
(23)	QB	A	9: 1	26,500	71
(24)	QB	D	9: 1	9,100	79
(25)	QB	A	2: 1	9,300	120
(26)	QB	A	9: 1	10,300	124
(27)	QB	A	9: 1	17,500	170

Table 39. Library of polymeric micelle particles selected to be tested in uptake studies; uptake by macrophages was compared to uptake by non-macrophages. Synthesised polymers were either pure-block (PB), copolymers purified before second polymerisation or quasi-block (QB), copolymers not purified. All polymers were fluorescently labelled with rhodamine B and crosslinked. Five different polymer block structures were made (**Figure 44**). Micelle diameters were measured using DLS in water.

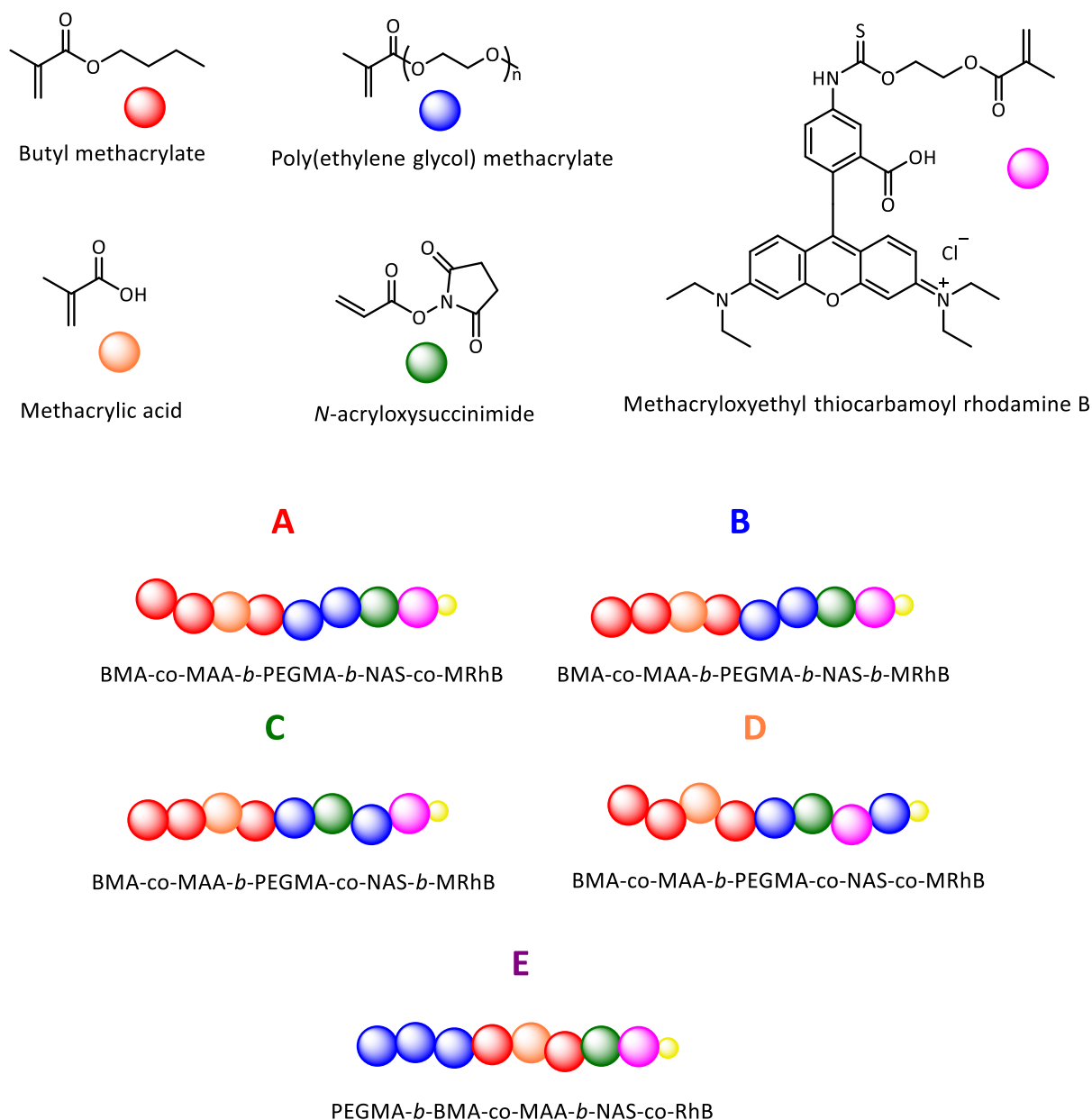


Figure 44. Different polymer block structures.

Micelles were initially tested using primary murine macrophages and non-macrophages harvested from bone marrow. The cell isolation protocol is detailed in the experimental section of this chapter. In later studies primary macrophages were harvested from the peritoneal cavity as well as from bone marrow and endocytosis by peritoneal macrophages was compared to endocytosis by bone marrow macrophages. Peritoneal macrophages are a better representation of target cells due to their activated state. However, significantly fewer can be harvested from the peritoneal cavity compared to the bone marrow, approximately 3.5×10^5 peritoneal macrophages and 7.5×10^6 bone marrow macrophages can be harvested

per mouse. A cell sorter was used to sort macrophages and non-macrophages from the harvested cell populations. Details of the labelling and sorting strategies are outlined in the experimental section of this chapter (4.4.4). Endocytosis of micelles by macrophages and non-macrophages was assessed using fluorescence activated cell sorting (FACS).

4.2.1 Determining micelle concentration for endocytosis studies

A dose response was performed to establish an appropriate concentration of polymer for uptake studies. Solutions of samples **(2)** and **(15)** (18 nm and 50 nm micelles respectively) were made up at concentrations of 1, 10, 25, 50, 75, 100 and 500 $\mu\text{g/ml}$. Micelle solutions were added to sorted bone marrow macrophages and non-macrophages to give final micelle concentrations of 0.17, 1.7, 4.2, 8.3, 13, 17 and 83 $\mu\text{g/ml}$. The cell solutions (200,000 cells/ml) were incubated at 37 °C for 30 min before micelle endocytosis was analysed. 37 °C was used as this is body temperature. Endocytosis was measured by the amount of rhodamine fluorescence detected in cells.

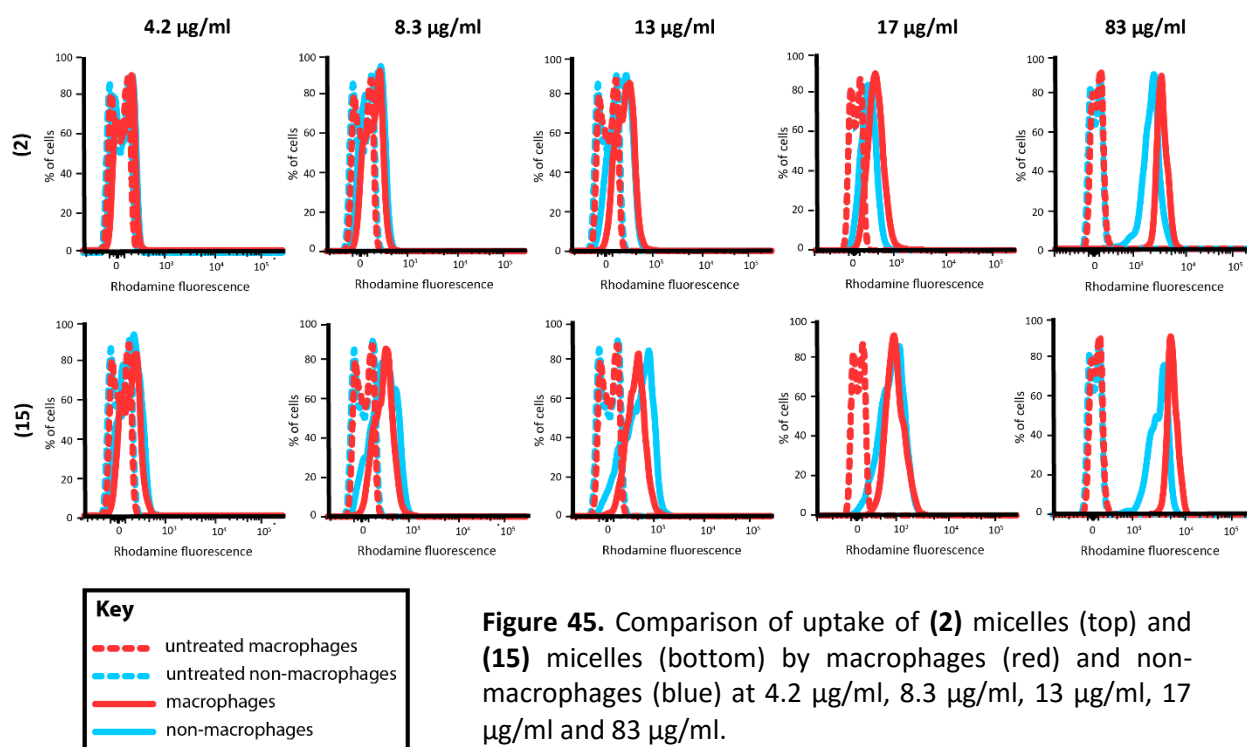


Figure 45. Comparison of uptake of **(2)** micelles (top) and **(15)** micelles (bottom) by macrophages (red) and non-macrophages (blue) at 4.2 $\mu\text{g/ml}$, 8.3 $\mu\text{g/ml}$, 13 $\mu\text{g/ml}$, 17 $\mu\text{g/ml}$ and 83 $\mu\text{g/ml}$.

Insignificant endocytosis of micelles was detected below concentrations of 8.3 $\mu\text{g/ml}$. At 13 $\mu\text{g/ml}$, significant endocytosis of micelle **(15)** occurred but not of **(2)**. At 17 $\mu\text{g/ml}$ a shift in

rhodamine fluorescence was detected for both micelles, **(15)** was endocytosed at least tenfold more than **(2)**. At 83 $\mu\text{g/ml}$ both micelles were readily endocytosed.

17 $\mu\text{g/ml}$ was determined to be the optimum concentration for comparison between **(2)** and **(15)**. To avoid concentration bias, the endocytosis of all micelles were tested at the same concentration, 17 $\mu\text{g/ml}$ was standardised as the concentration to be used in all endocytosis studies.

4.2.2 Determining optimum incubation time

To determine the optimum incubation time for the endocytosis assays, different time points were studied. A 2 μm , Pacific Blue labelled, latex bead (zwitterionic polystyrene microspheres) positive control was used for this study as it is known to be phagocytosed by primary macrophages. Three incubation times were selected for the study: 30, 60 and 90 min. There was no increase in the number of cells which internalised the latex beads, or in the number of latex beads internalised by cells, between 30 and 90 min. It was therefore determined that 30 min was sufficient for incubation, this time was used in all further studies.

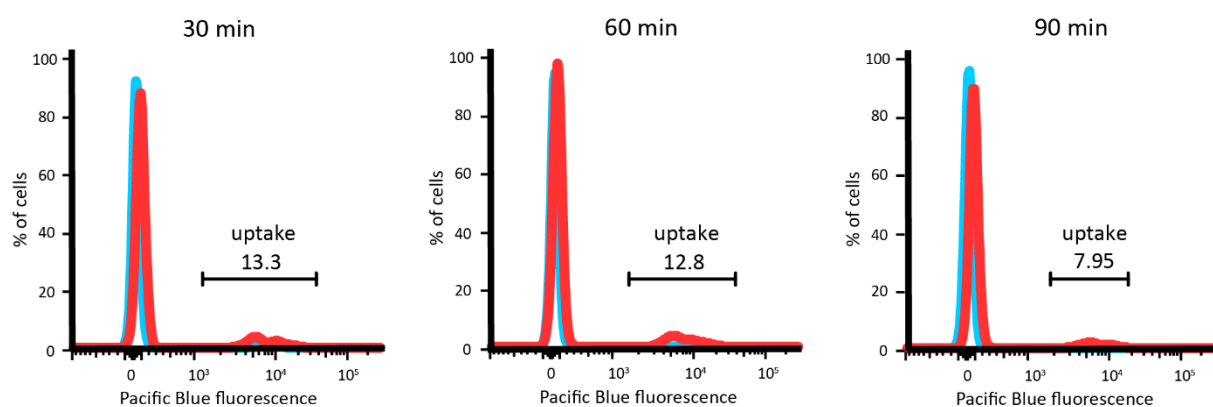


Figure 46. Uptake levels after incubation for 30, 60 and 90 min from left to right. No increase in phagocytosis was seen after 30 min.

4.2.3 Initial screening of micelles

The library of 27 micelle particles was screened to determine the effect of micelle size on endocytosis. Each sample was incubated separately with macrophages and non-macrophages, and the internalisation of micelles by the different cell types was compared using FACS analysis. As not all screening experiments could be carried out on one day, mean channel fluorescence (MCF) values were normalised using one sample tested on

multiple days. To prevent any differences in sample rhodamine fluorescence being identified as false positives, endocytosis was normalised by dividing MCF values by the fluorescence of each sample (determined in **Chapter III**); these results are displayed as 'rh normalised endocytosis' in **Table 40**.

Micelle	Micelle size in PBS (nm)	Normalised macrophage MCF	Normalised non-macrophage MCF	Macrophage rh normalised endocytosis	Non-macrophage rh normalised endocytosis	Fold change
(1)	14	762	548	1.76	1.27	0.39
(2)	190*	868	446	0.38	0.19	1.00
(3)	15	2,510	1,840	1.03	0.75	0.37
(4)	28	4,920	2,160	2.53	1.11	1.28
(5)	12*	3,120	1,330	1.84	0.78	1.36
(7)	25	12,700	6,840	3.66	1.97	0.86
(8)	37	737	432	0.74	0.43	0.72
(9)	54	8,900	4,240	4.05	1.93	1.10
(10)	31	863	648	1.14	0.86	0.33
(11)	80	5,860	2,570	2.12	0.93	1.28
(12)	46	633	511	1.99	1.61	0.24
(14)	63	686	616	0.84	0.75	0.12
(15)	45	1,910	1,360	1.55	1.10	0.41
(16)	54	725	613	1.03	0.87	0.18
(17)	53	647	350	1.26	0.68	0.85
(18)	73	637	547	0.97	4.12	-0.76
(20)	53	650	541	0.76	0.63	0.21
(21)	35	715	575	1.20	0.97	0.24
(22)	54	3,280	2,710	2.46	2.03	0.21
(24)	58	2,220	908	2.84	1.16	1.45
(25)	51	6,250	2,710	8.74	3.79	1.31
(26)	44	2,220	1,770	1.01	0.81	0.25
(27)	57	475	382	0.94	0.75	0.25

Table 40. Micelle size compared to endocytosis by macrophages and non-macrophages. Endocytosis displayed as MCF. MCF values were normalised to allow a direct comparison between samples tested on different days. Endocytosis normalised with regards to sample rhodamine (rh) fluorescence (calculated using sample fluorescence values, detailed in appendices, **Table 51**) is also displayed. *Samples showed two DLS peaks, indicating two different particle sizes. The main peak is listed. DLS analysis was performed using a Malvern Zetasizer.

The MCF of untreated cells used as a negative control in all experiments and was < 150. MCF values for all samples were well above 150, establishing that micelles were successfully endocytosed. The MCF values for different micelles vary significantly, suggesting varying levels of endocytosis.

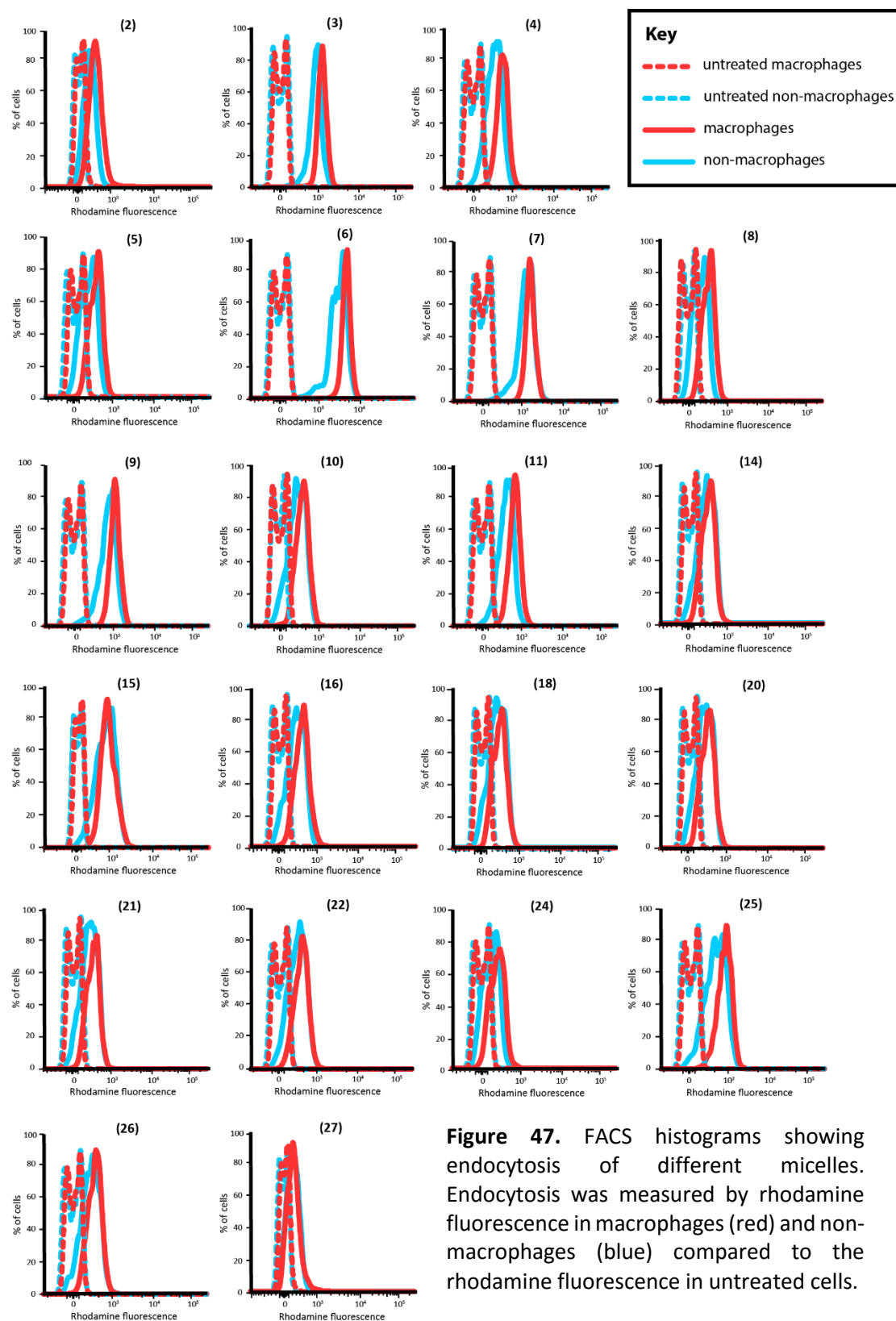


Figure 47. FACS histograms showing endocytosis of different micelles. Endocytosis was measured by rhodamine fluorescence in macrophages (red) and non-macrophages (blue) compared to the rhodamine fluorescence in untreated cells.

Figure 47 shows a collection of FACS histograms from the screening study. The histograms include untreated cells for comparison. All cells (macrophages and non-macrophages)

endocytosed some of the micelles, however, some micelles e.g. **(25)** were endocytosed more by macrophages than by non-macrophages. It should be noted that the histogram plots have not been normalised, as they are a direct representation of the experimental data. In comparisons between micelles, the data in **Table 40** was used; this data was normalised to allow for variations between experiments on different days and normalised with regards to rhodamine fluorescence.

No visible shift in rhodamine fluorescence, compared to untreated cells, was observed in histograms for micelles **(1)**, **(12)**, **(17)** and **(19)**, these micelles were internalised by cells but only in low quantities. Therefore, the histograms for these samples were not included in **Figure 47**. The following samples did not fully dissolve in PBS and so it was not possible to test their uptake: **(11)**, **(19)** and **(23)**, these samples were excluded from the screening study.

To provide further proof of the internalisation of micelles, fluorescence microscopy studies were performed. As in FACS studies, micelles (17 µg/ml) were incubated with sorted macrophages (200,000 cells/ml) for 30 min at 37 °C. Rhodamine fluorescence can clearly be seen inside the macrophages in **Figure 48**. If micelles coated the cells but had not being internalised a 'halo' of fluorescence would be seen around the edge of the cell.

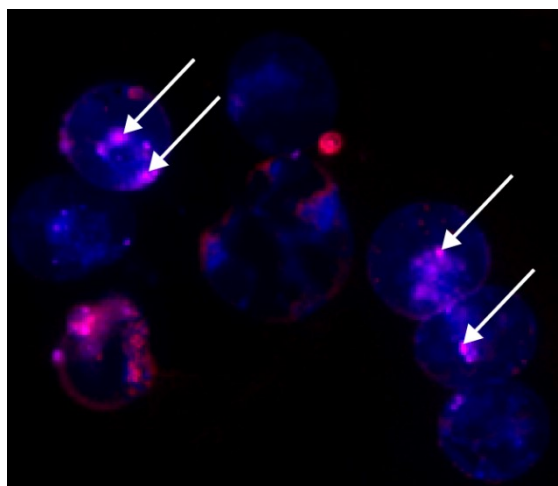


Figure 48. Fluorescent microscope image; Rhodamine B labelled micelle particles (pink) can be seen to have been internalised by macrophages, (blue, BV510 fluorescence). Macrophages were labelled with CD45-BV510 to enhance their visibility under the fluorescence microscope.

The differences in endocytosis of different micelles at the fixed time point and concentration studied was not size dependent within the size range tested. Micelle size did not correlate with endocytosis (**Figure 49**); linear regression (R) values for micelle size versus endocytosis were 0.15 for macrophages and 0.07 for non-macrophages. This finding does not correspond with published work.^{257,258} This is possibly due to the micelle size range being too small to

have a significant effect on endocytosis. As discussed in detail in **Chapter 1**, particle size has commonly been found to affect the rate and mechanism of internalisation by cells.^{63,96–98} In phagocytosis large particles are engulfed while in other types of endocytosis, such as receptor mediated endocytosis, smaller particles and molecules are internalised when small pits are formed on the cell surface. Endocytosis mechanisms are outlined in more detail in **4.1.1**.

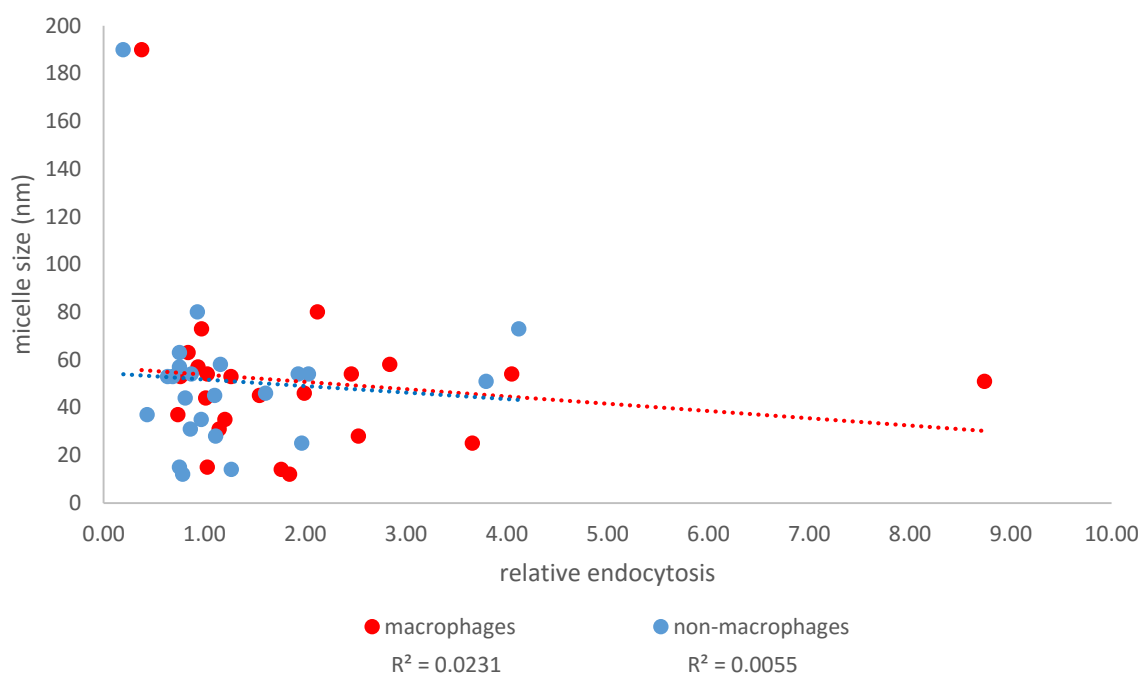


Figure 49. Plot of micelle size against endocytosis of micelles by macrophages (red) and non-macrophages (blue) shows no correlation between size and endocytosis for either cell type. Relative endocytosis was calculated using FACS MCF values which were standardised and normalised in terms of polymer rhodamine fluorescence.

Although there was no correlation between size and endocytosis, different micelle samples were endocytosed varying amounts. To investigate this result further and determine if it was significant, five micelle samples with high levels of endocytosis and two with low levels of endocytosis were selected for further studies (described and discussed in **4.2.6**). The samples selected were **(3)**, **(7)**, **(9)**, **(15)** and **(25)** (high levels of endocytosis) and **(12)** and **(17)** (low endocytosis).

4.2.4 Method of uptake

All cells in the body endocytose, whereas only macrophages, and a few other cell types, are capable of phagocytosis. By comparing macrophage and non-macrophage uptake profiles

information about the type of uptake occurring can be determined. The results of the initial screening of the library of micelle particles verified that micelles were endocytosed but that no (or possibly very low levels of) phagocytosis was involved.

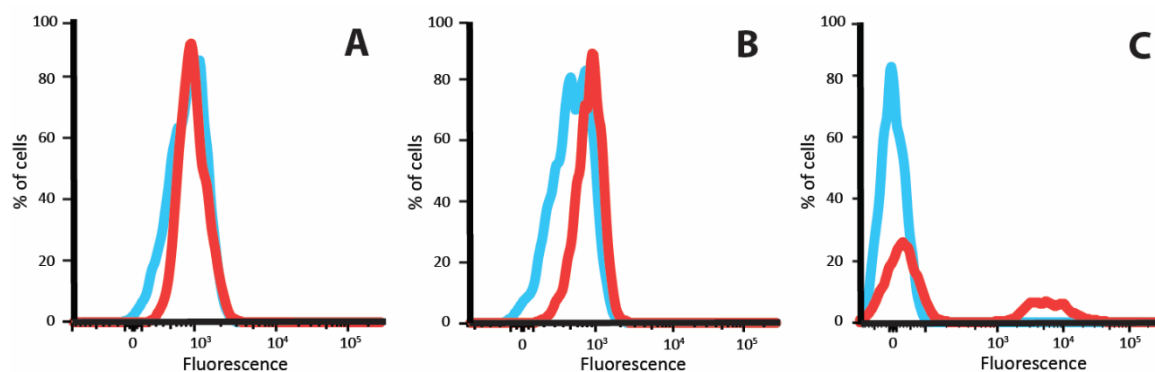


Figure 50. Uptake profiles of micelles (A and B) and latex beads (C) by macrophages (red) and non-macrophages (blue). A is a histogram showing the uptake of micelle (15) and B is a histogram showing the uptake of micelle (25).

Figure 50 shows examples of three different uptake profiles. In graph A uptake of micelle (15) by macrophages and non-macrophages was very similar. This indicates that no phagocytosis is occurring as non-macrophages are not capable of phagocytosing. In graph B uptake of micelle (25) by macrophages is higher than uptake by non-macrophages. This could either signify a mixture of phagocytosis and other types of endocytosis by macrophages or macrophages could be endocytosing *via* the same (non-phagocytic) mechanism(s) as non-macrophages, but more efficiently. Graph C is an example of phagocytic uptake of 2 μm latex beads which are too large to be endocytosed by other endocytosis mechanisms; only macrophages phagocytose the beads. All micelles tested had similar macrophage and non-macrophage uptake profiles, indicating that phagocytosis is not the method of uptake. To achieve selective delivery to macrophages larger micelles, which can only be phagocytosed, need to be synthesised. As shown in Graph C, 2 μm is large enough to get selective phagocytosis of latex beads. Uptake studies using different sizes of latex beads were performed to determine more information about the size required to get selective phagocytosis, see section 4.2.9.

4.2.5 Activated macrophages

Initial screening of micelles was carried out using bone marrow cells. Bone marrow macrophages are un-activated. Activated macrophages can be found in the peritoneal cavity.

Activated macrophages are a better representation of target cells so peritoneal cells were included in further studies when possible.

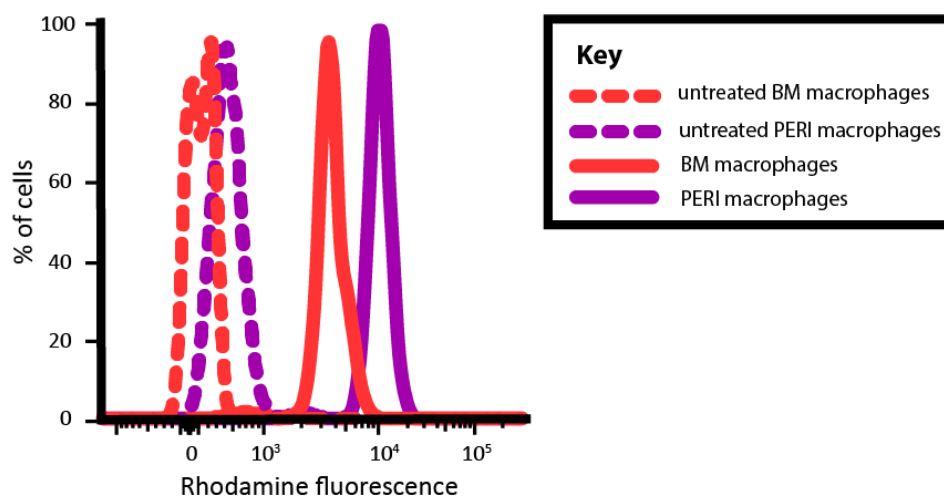


Figure 51. Comparison of endocytosis profiles of a 73 nm micelle by bone marrow (BM) and peritoneal (PERI) macrophages treated under the same conditions. Fluorescence profiles of untreated macrophages are also displayed.

Figure 51 shows peritoneal macrophages endocytosed more micelles than bone marrow macrophages when incubated with 73 nm micelles for 30 min at 37 °C. Micelles were used at a concentration of 17 µg/ml and sorted cells at a concentration of 200,000 cells/ml. Cells were analysed using flow cytometry at a concentration of 1×10^6 cells/ml.

4.2.6 Investigation into significant results

As discussed previously, screening a selected library of synthesised micelles identified a range of different levels of micelle endocytosis. Five micelles with high levels of endocytosis and two with low levels of endocytosis were selected to be of interest for further studies. Micelles with high endocytosis levels show great promise for drug delivery purposes and so are of particular interest. **Table 41** summarises the micelles used in this study and their initial screening results.

Micelle	Polymer type	BMA: MAA ratio	Polymer MW (Da)	Micelle diameter (nm)	Macrophage endocytosis	Non-macrophage endocytosis	Fold change
(3)	Quasi-tetrablock	9: 1	7,700	18	1.03	0.750	0.37
(7)	Pure-triblock	4: 1	6,500	27	3.66	1.97	0.86
(9)	Pure-triblock	9: 1	7,900	40	4.05	1.93	1.10
(12)	Quasi-triblock	2: 1	7,700	46	1.99	1.61	0.24
(15)	Quasi-triblock	9: 1	9,200	50	1.55	1.10	0.41
(17)	Quasi-triblock	4: 1	8,700	53	1.26	0.680	0.85
(25)	Quasi-triblock	2: 1	9,300	120	8.74	3.79	1.31
(26)	Quasi-triblock	9: 1	10,300	124	1.01	0.810	0.25

Table 41. Details of micelle samples selected for further studies. Micelle diameter was measured in PBS to simulate in vitro experiment conditions. Macrophage and non-macrophage endocytosis values are in RFU and were calculated using FACS MCF values that were standardised and then normalised in terms of polymer rhodamine fluorescence.

Reproducibility of results

To determine the reproducibility of significant results, 3 biological repeats quantifying endocytosis of selected micelles were performed. Experiments were carried out on different days with cells harvested from different mice. At least 3 mice were used per experiment and the cells were pooled before being labelled and then divided into incubation tubes.

Micelle	n = 1 MCF value	n = 2 MCF value	n = 3 MCF value
(3)	3,280	9,390	9,720
(7)	13,400	50,800	51,000
(9)	6,520	18,000	16,700
(12)	1,770	4,920	5,440
(15)	2,000	5,440	5,610
(17)	n/a	2,270	2,630
(25)	27,600	43,000	47,900

Table 42. MCF values for 7 samples tested in experiments. Results shown are for peritoneal macrophages. Bone marrow macrophages and non-macrophages were also tested; the same trends were seen for all cell types. Micelle (17) was not included in n = 1 experiment. Micelles were incubated with cells for 30 min at 37 °C. Micelle concentration: 17 µg/ml, cell concentration: 200,000 cells/ml.

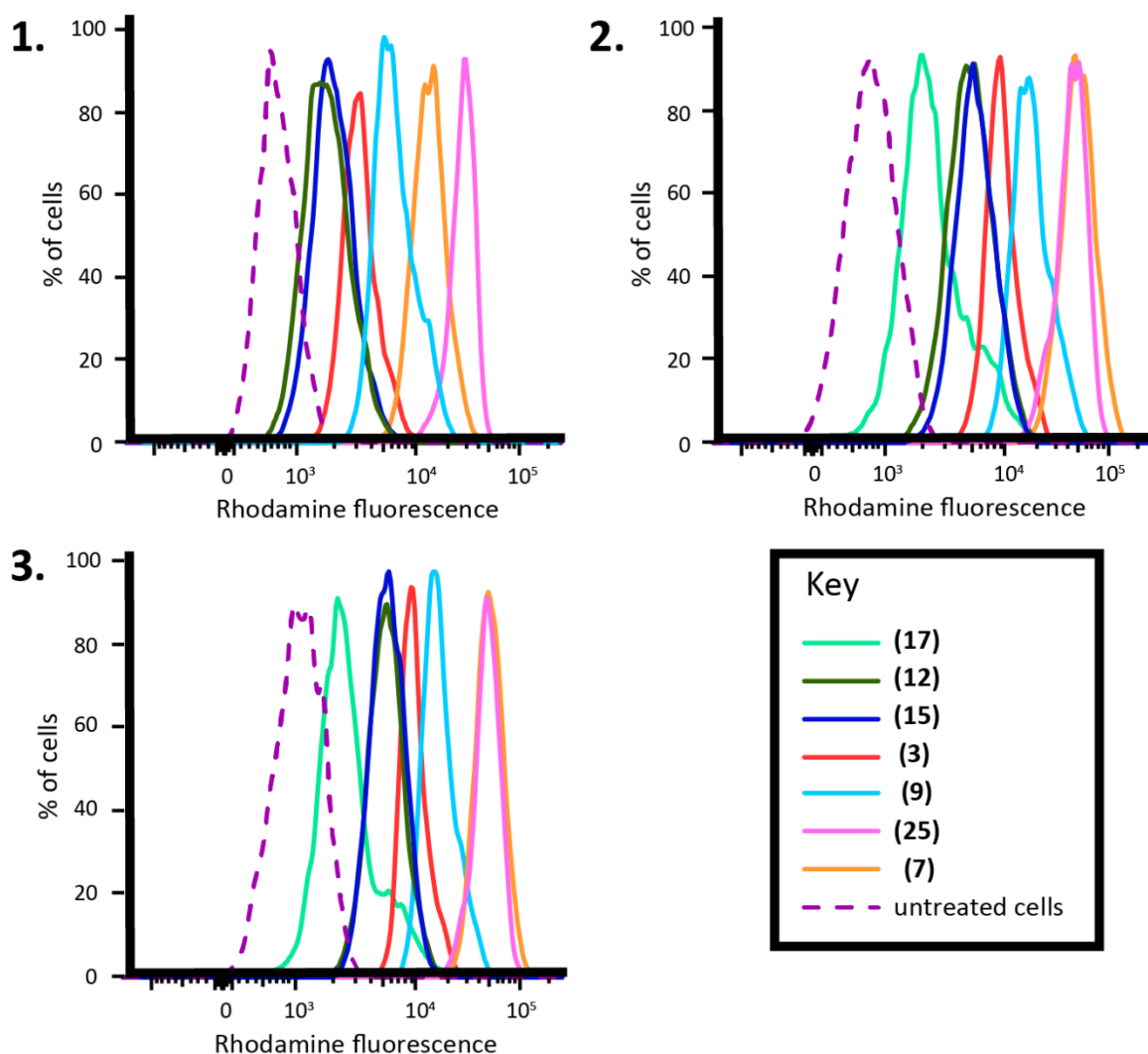


Figure 52. Histograms showing results from $n = 3$ experiments determining result repeatability (data shown in **Table 42**). Results shown are for peritoneal macrophages. Bone marrow macrophages and non-macrophages showed the same trends. Micelle **(17)** was not included in $n = 1$ experiment.

Micelle endocytosis varied between experiments but trends were consistent: endocytosis of **(7) $\approx$ (25) > (9) > (3) > (12) $\approx$ (15) > (17)**. The endocytosis of one micelle in relation to another remained the same, confirming the reproducibility of the results. The variation in endocytosis between experiments highlights the importance of normalising results from different experiments before direct comparisons are carried out.

Quantifying the results

As micelle endocytosis was quantified by rhodamine fluorescence the amount of rhodamine per micelle had to be determined in order to fully quantify uptake.

Figure 53 illustrates why it is important to quantify endocytosis of micelles and not just quantify rhodamine fluorescence. The same amount of fluorescence would be detected if the micelles in **A**, **B** or **C** were endocytosed. Differences in rhodamine: polymer chain ratios between samples and differences in the number of polymer chains per micelle could affect the results. To account for these differences the number of polymer chains per micelle was determined. Using this value, and the relative polymer fluorescence of each sample, it was possible to calculate the relative endocytosis of different micelles. Relative micelle fluorescence = relative polymer fluorescence x no. polymer chains per micelle. Normalised micelle endocytosis = macrophage MCF/ relative micelle fluorescence.

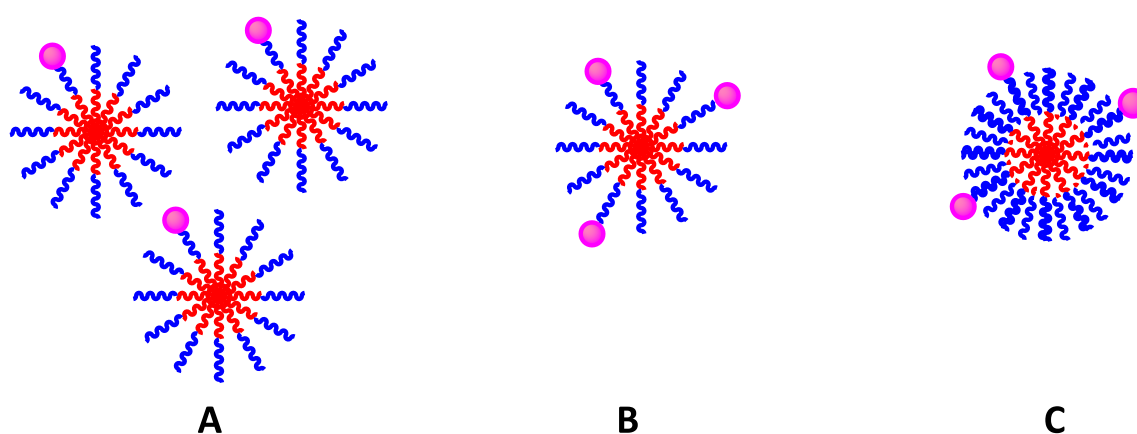


Figure 53. Three different micelle scenarios which would all result in the same amount of ‘endocytosis’ being detected as the same amount of rhodamine is present in A, B and C.

Relative polymer fluorescence was determined by plotting fluorescence versus concentration for each sample, an example for sample **(3)** is shown in **Figure 54**. The equation determined from the line of best fit was used to calculate the relative polymer fluorescence for each sample from the cell uptake studies. The relative polymer fluorescence values for samples; **(3)**, **(9)**, **(7)**, **(12)** and **(15)** are displayed in **Table 44**. A more detailed description of how relative polymer fluorescence was calculated and values for all samples can be found in **Chapter III**.

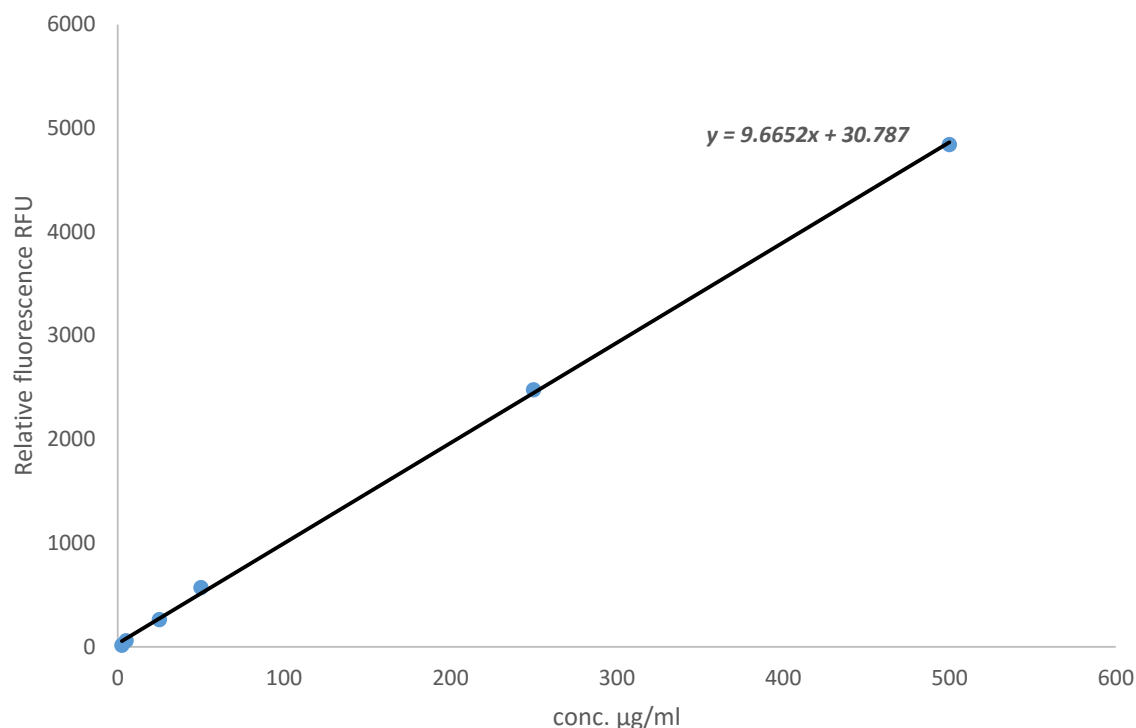


Figure 54. Plot of rhodamine fluorescence versus concentration for sample **(3)**. Equation determined from the line of best fit was used to calculate the relative fluorescence intensity of the sample.

The number of polymer chains per micelle was determined using multi-angle light scattering (MALS). MALS analysis uses a sample's refractive index increment (dn/dc) to calculate the molecular weight of a micelle at a known concentration. Once a micelle's molecular weight is known, the number of chains per particle can be calculated: micelle molecular weight/ polymer molecular weight = no. of chains. Large sample quantities were required for MALS analysis and samples had to be soluble in water at concentration of 6 mg/ml. This meant that it was not possible to analyse all samples. Results of analysed samples are displayed in **Table 43**.

Micelle	dn/dc (ml/g)	Micelle M_n (Da)	Polymer M_n (Da)	Chains per micelle
(1)	0.123	2.935×10^5	7,400	39
(3)	0.113	3.366×10^5	7,700	44
(5)	0.142	1.717×10^5	6,300	27
(7)	0.138	3.002×10^5	6,500	46
(9)	0.132	7.052×10^5	7,900	89
(12)	0.124	1.380×10^5	7,700	18
(15)	0.144	3.381×10^5	9,200	37

Table 43. Refractive index increment (dn/dc) and multi-angle light scattering (MALS) results. Micelle M_n / polymer M_n = no. of polymer chains per micelle.

The relative micelle endocytosis of selected micelles analysed in the repeatability study were calculated, see **Table 44**. Once rhodamine fluorescence per micelle was taken into account endocytosis of **(12)** > **(7)** > **(3)** $\approx$ **(9)** $\approx$ **(15)**. It should be noted that sample **(25)** could not be included in this comparison as it was not soluble at high concentrations and therefore could not be analysed *via* MALS.

Micelle	Relative polymer fluorescence	No. polymer chains per micelle	Relative micelle fluorescence	Macrophage MCF	Relative micelle endocytosis
(3)	7.54	44	332	3,280	9.90
(7)	9.05	46	416	13,400	32.3
(9)	6.94	89	618	6,520	10.6
(12)	0.980	18	18.0	1,770	98.4
(15)	4.56	37	169	2,000	11.9

Table 44. All fluorescence and endocytosis values are in RFU. Relative polymer fluorescence is the relative fluorescence per mole of polymer and relative micelle fluorescence is the relative fluorescence per mole of micelles. Relative micelle fluorescence = relative polymer fluorescence x no. polymer chains per micelle. Normalised micelle endocytosis = macrophage MCF/ relative micelle fluorescence. Macrophage MCF values are peritoneal macrophage results from experiment 1 of the reproducibility study.

4.2.7 Sorted cells versus unsorted cells

An assay was performed to determine whether unsorted cells could be used instead of sorted cells. It is important to use sorted cells when doing microscopy work, to get clear images the slide needs to contain a large number of the cells of interest. For flow cytometry purposes however, it is not necessary to start with a sorted population as the cells of interest can be identified *via* gating after the events have been collected. As cell sorting can be time consuming and is expensive, an assay was performed to determine if results were reproducible when cells were not sorted prior to incubation. Micelle samples **(3)** and **(8)** were incubated with sorted and unsorted bone marrow and peritoneal cells. The endocytosis profiles for sorted and unsorted cells were the same, see example in **Figure 55**. It was decided that cells would not be sorted prior to incubation unless the cells were going to be analysed using fluorescence microscopy.

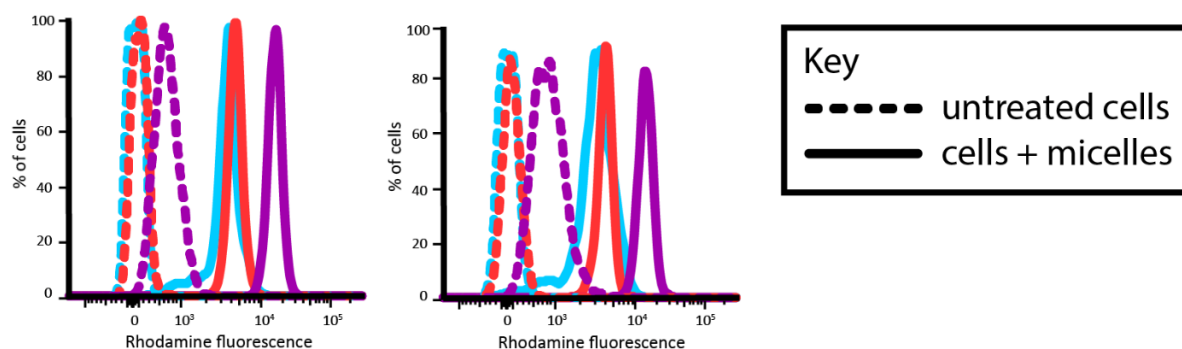


Figure 55. Endocytosis of **(3)** by sorted cells (left) and unsorted cells (right). Results are displayed for bone marrow macrophages (red), peritoneal macrophages (purple) and bone marrow non-macrophages (blue).

4.2.8 Effect of crosslinking on particle endocytosis

A comparison of endocytosis of crosslinked and uncrosslinked micelles was performed. Four different types of micelle were used in the study and endocytosis of micelles by both bone marrow and peritoneal cells was determined.

Micelles were crosslinked *via* the incorporation of *N*-acryloxysuccinimide (NAS) into the PEGMA block of the BMA-co-MAA-*b*-PEGMA block copolymer, followed by crosslinking of the NAS units using a primary diamine, as detailed in **Chapter III**, section **3.2.2**.

The endocytosis of uncrosslinked micelles was higher than that of crosslinked micelles, results shown in **Figure 56** and **Table 45**. It is possible that uncrosslinked micelles are disassembling *in vitro* and are internalised as polymer chains opposed to as micelles. As single polymer chains are smaller than assembled micelle particles, the polymer chains would potentially be able to be pinocytosed. This would explain the difference in endocytosis levels between crosslinked and uncrosslinked micelles. Assembled micelles were too large for pinocytosis and so were internalised *via* other endocytosis mechanisms but not by pinocytosis, while uncrosslinked micelles were taken up *via* pinocytosis and other endocytosis mechanisms.

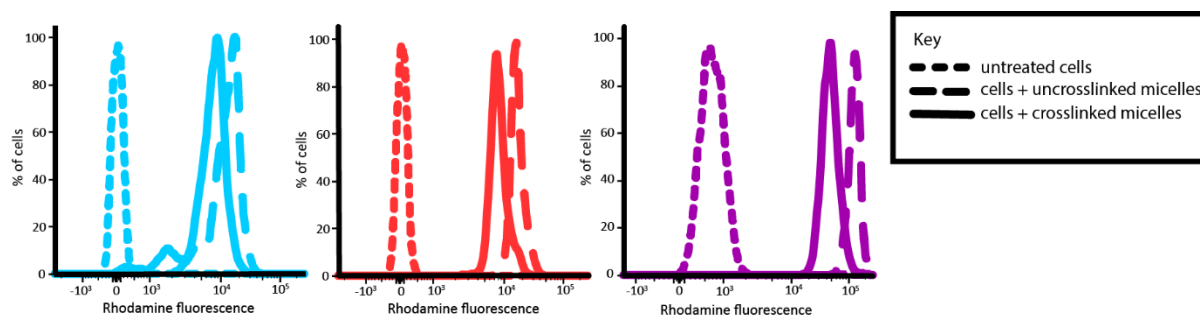


Figure 56. Example of comparison between uncrosslinked and crosslinked micelle endocytosis. Results shown are for micelle (25). From left to right the plots represent bone marrow non-macrophages (blue), bone marrow macrophages (red) and peritoneal macrophages (purple).

Micelle	Size of uncrosslinked micelle (nm)	Size of crosslinked micelle (nm)	Uncrosslinked micelle endocytosis (MCF)	Crosslinked micelle endocytosis (MCF)	Fold change
(7)	28	25	68,400	48,400	0.41
			16,900	8,560	0.97
			12,000	6,560	0.82
(18)	45	73	9,140	3,330	1.74
			3,310	945	2.50
			2,710	993	1.73
(25)	25	51	12,600	90,200	-0.86
			24,700	5,690	3.34
			12,600	3,530	2.57
(27)	103	57	12,800	2,800	3.57
			1,620	622	1.60
			1,980	718	1.76

Table 45. Peritoneal macrophage endocytosis results shown in purple, bone marrow macrophage endocytosis results shown in red and bone marrow non-macrophage endocytosis results shown in blue. An increase in endocytosis was observed for uncrosslinked micelles in comparison to crosslinked micelles.

4.2.9 Determining optimum size to selectively target macrophages

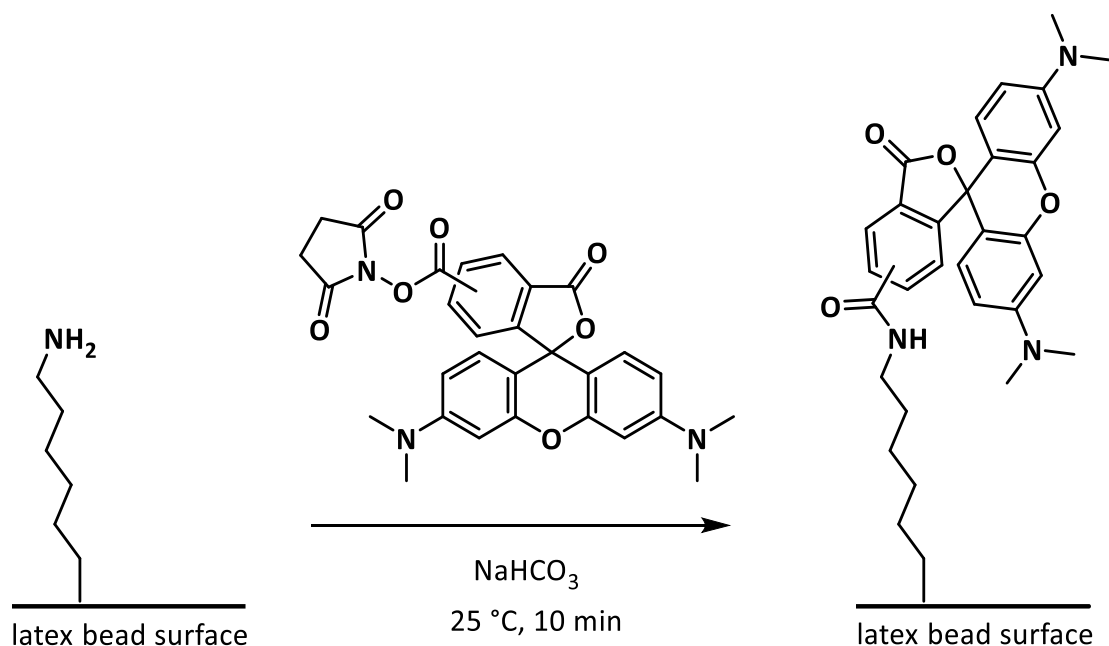
Micelles synthesised and tested in this project were not large enough to be selectively phagocytosed by macrophages. The synthesis of micelles that are too large to be endocytosed by non-phagocytic mechanisms would enable macrophages to be selectively targeted. Determining the size that particles become selectively phagocytosed will be useful for future micelle design in work to optimise the drug delivery system.

To investigate the size cut-off point for non-phagocytic endocytosis, 1 μm , 400 nm and 60 nm rhodamine labelled latex beads were incubated with macrophages and non-macrophages for 30 min at 37 $^{\circ}\text{C}$. 2 μm latex beads, used as a positive control for phagocytosis in all uptake

studies in this project, are phagocytosed by macrophages but are too large to be endocytosed by non-macrophages. The uptake of the latex beads by macrophages and non-macrophages was studied using flow cytometry.

Labelling latex beads

The latex beads used had a high density coating of amine groups, attached *via* an aliphatic six-carbon spacer arm. This amine functionality was used to label the beads with rhodamine *via* a NHS-coupling reaction. Excess NHS-rhodamine was removed by dialysis. During the labelling, the latex beads changed from colourless to fluorescent pink. Bead fluorescence was confirmed using a UV lamp and by FACS analysis for 1 μm beads (60 and 400 nm beads were too small to be detected as events when passed through the analyser).



Scheme 19. Aliphatic amine coated latex beads were labelled using NHS-functionalised rhodamine.

Uptake of latex beads

As 2 μm latex beads were used in all experiments as a phagocytosis positive control, the protocol for their use was well established. Cells were incubated with latex beads at a concentration of 1.7×10^6 beads/ml for 30 min, histogram displayed in **Figure 57**. However, when these conditions were used to study endocytosis of 1 μm , 400 nm and 60 nm rhodamine labelled latex beads, no bead internalisation was detected.

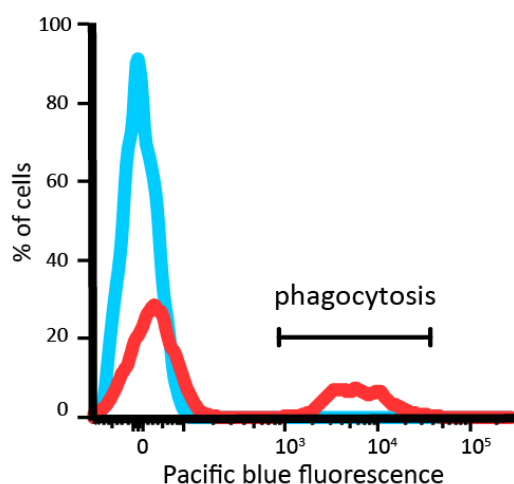


Figure 57. 2 μm latex beads, labelled with Pacific Blue, were phagocytosed by macrophages (red) but not endocytosed by non-macrophages (blue). Cells treated with 2 μm latex beads at a concentration of 1.7×10^6 beads/ml (bead concentrations are approximate).

As no endocytosis was detected for 400 and 60 nm beads at a concentration of 1.7×10^6 beads/ml, a series of higher concentrations, 1.7×10^7 , 1.7×10^8 and 1.7×10^9 beads/ml, were tested (**Figure 58**). It was determined that the smaller the bead size, the higher the bead concentration needed to be to result in a detectable level of endocytosis. Endocytosis of 60 nm beads was not detected at any of the concentrations tested. The fact that smaller beads were less fluorescent could have contributed to this result. The surface of the beads was labelled with a fluorescent compound (either Pacific Blue or rhodamine), smaller beads had a smaller surface area and therefore were less fluorescent. It is also possible that smaller beads were endocytosed less than larger ones. Bead concentration was calculated by bead number; bead concentration by volume (or mass) is higher for larger beads so the bead concentration by volume (or mass) was higher when larger beads were used. This would explain the increase in endocytosis with bead size.

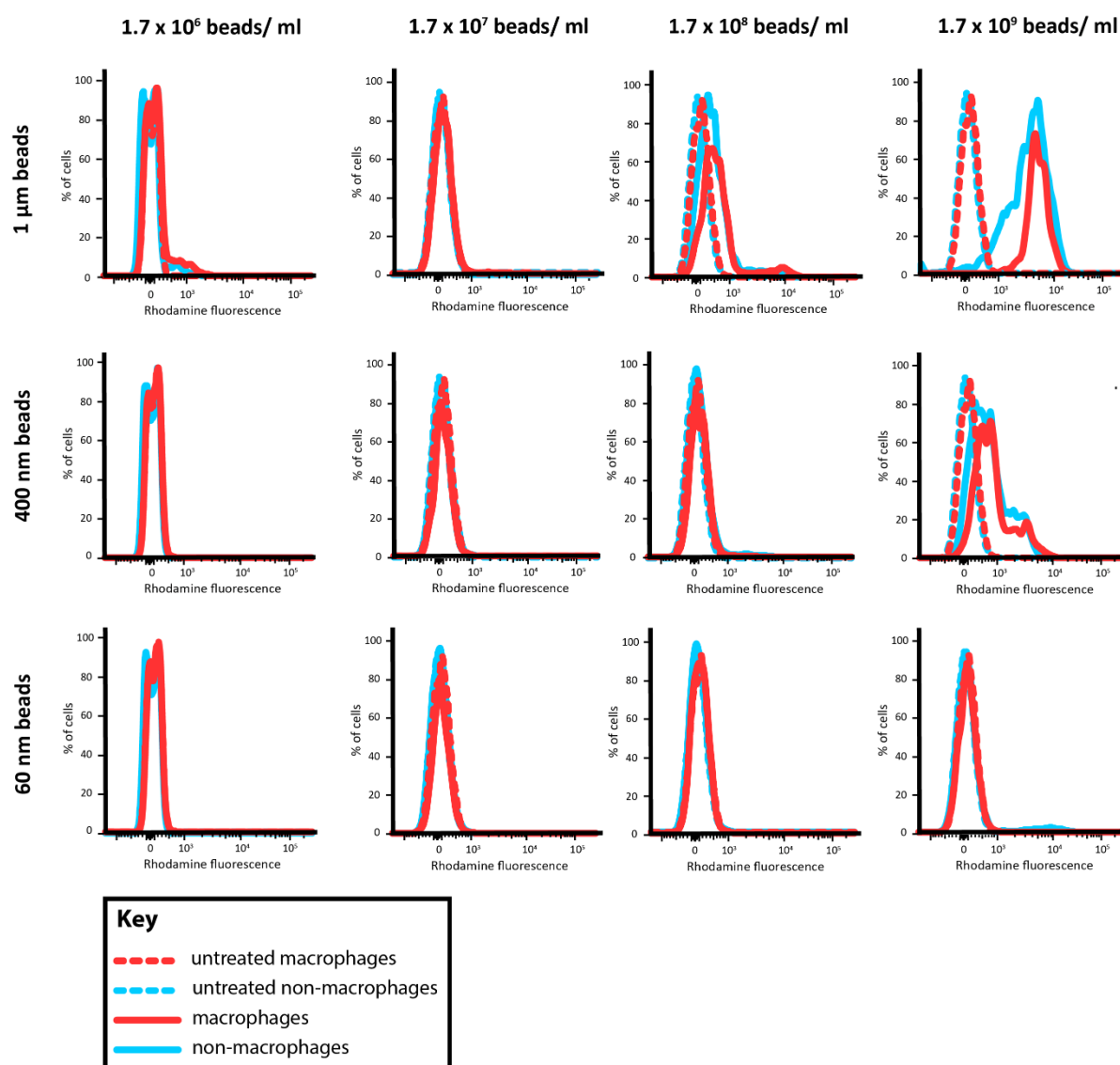


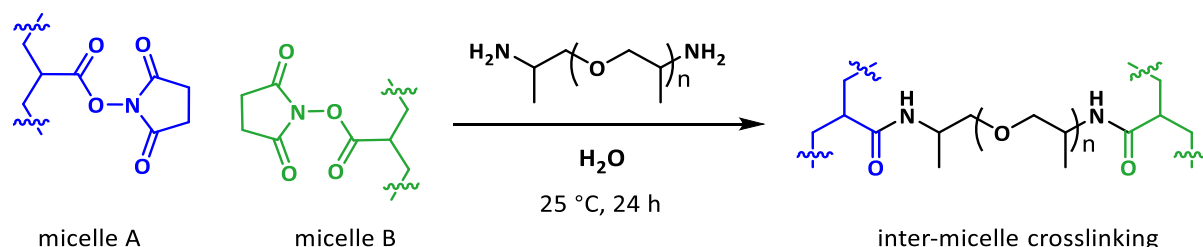
Figure 58. Histograms showing the endocytosis of 1 µm, 400 nm and 60 nm latex beads (labelled with rhodamine) after incubation at concentrations of 1.7×10^6 , 1.7×10^7 , 1.7×10^8 and 1.7×10^9 beads/ml.

Although no endocytosis was detected for 60 nm beads, 400 nm and 1 µm bead endocytosis profiles were obtained at 1.7×10^9 beads/ml. The uptake profiles of 400 nm and 1 µm latex beads by macrophages and non-macrophages were very similar, indicating that phagocytosis was not the mechanism of uptake. As it has been shown that phagocytosis is the mechanism of uptake for 2 µm latex beads it can be assumed that the size-cut off point for non-phagocytic endocytosis is between 1 and 2 µm. Micelle particles and latex beads have different structures so mechanistic results concerning latex bead internalisation will not necessarily correspond with micelle results. However, this result doubtlessly provides useful information about using size to selectively target the phagocytosis mechanism. It is unlikely for example, that if

non-macrophages can endocytose 1 μm latex beads *via* non-phagocytic mechanisms that they would not endocytose 1 μm micelles *via* the same mechanisms.

4.2.10 Inter-micelle crosslinking

Larger micelle particles were required to achieve selective delivery to macrophages. An attempt was made to form large particles by crosslinking micelles together. Intra-micelle crosslinking was previously done at very low concentrations to prevent inter-micelle crosslinking from occurring. In this experiment, the concentration of micelle in the crosslinking reaction was increased to try and cause inter-micelle crosslinking. The aim was to crosslink 5-10 micelles together to get particles $> 1 \mu\text{m}$. Apart from polymer concentration, reaction conditions remained the same as for intra-micelle crosslinking.



Scheme 20. Inter-micelle crosslinking between two different micelles.

The polymer concentrations tested were 1.4×10^{-5} , 1.6×10^{-4} and $3.1 \times 10^{-4} \text{ mol/dm}^3$. Unfortunately, inter-micelle crosslinking did not occur at the concentrations investigated. Increasing the concentration further may have resulted in the desired outcome but it is likely that at high concentrations the majority of micelles in solution would become inter-crosslinked and a gel would be formed.

4.2.11 Encapsulation of DAPI dye

Micelles had been found to be successfully internalised by macrophages but uptake of a delivery system is not analogous with delivery of a cargo. To determine the micelle's potential for encapsulation and cargo delivery, a dye was diffused into the micelles and then the cellular uptake of the loaded micelles was studied. A fluorescent blue dye, 4',6-diamidino-2-phenylindole (DAPI), was selected for encapsulation in micelles. DAPI is a common cell staining dye but it is normally used to stain dead cells as it cannot permeate living cell membranes unless used at high concentrations (25 $\mu\text{g/ml}$).²⁵⁹ DAPI was selected for

encapsulation in micelles for three reasons, i) it has basic functionality, making it more likely to be successfully encapsulated and retained inside the acidic micelle core. ii) DAPI cannot permeate live cell membranes on its own so any DAPI fluorescence detected in cells had to be a result of successful encapsulation and delivery. iii) The fluorescence profiles of rhodamine and DAPI do not overlap and so fluorescence of the two dyes can be detected and analysed separately but simultaneously.

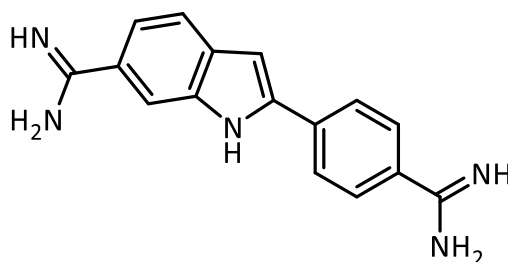


Figure 59. DAPI (4',6-diamidino-2-phenylindole).

DAPI was encapsulated in micelles via diffusion. Micelles were dissolved in water and a concentrated DAPI solution (5 mg/ml) was added. The reaction mixture was vortex mixed for 1 minute and then stirred for 24 h. It was hypothesised that the interaction between the basic dye and the acidic core of the micelle would enable the dye to be stably encapsulated. Micelles containing encapsulated DAPI were dialysed to remove excess DAPI. After dialysis, the DAPI fluorescence of micellar solutions was measured. As DAPI fluorescence and concentration have a linear correlation (as shown in **Figure 60**), the solution fluorescence could be used to calculate the concentration of DAPI encapsulated in the micelles. Different micelles encapsulated different quantities of DAPI.

DAPI concentration ($\mu\text{g/ml}$)	Fluorescence (RFU)
0.0130	1,640
0.0640	1,710
0.320	1,960
1.60	3,310
8.00	8,290
40.0	20,200
200	27,700
1,000	26,300

Table 46. DAPI fluorescence at various concentrations. Fluorescence values are an average of $n = 3$ measurements and are displayed in RFU. Measurements were taken in PBS. DAPI Ex_{max} : 358 nm, Em_{max} : 462 nm.

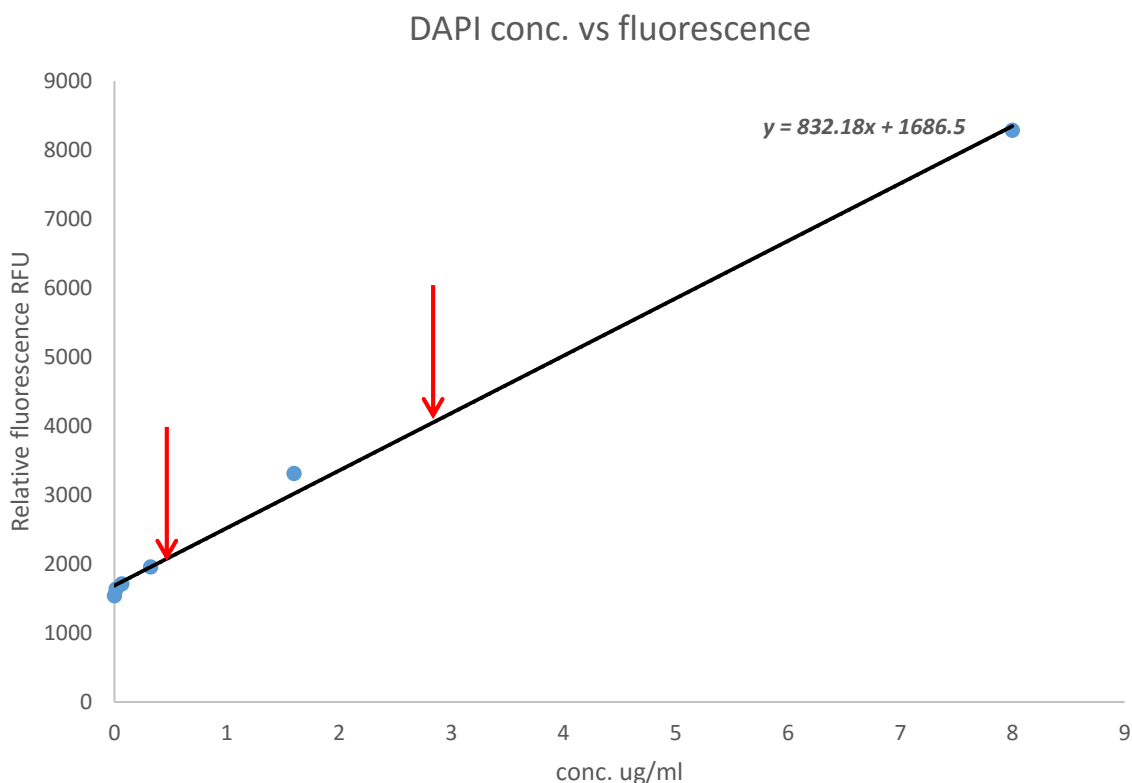


Figure 60. Plot of DAPI fluorescence versus concentration. Line of best fit used to determine concentration of DAPI encapsulated inside different micelles. Red arrows highlight the lowest concentration of DAPI encapsulated (0.19 µg/ml- (17)) and the highest concentration encapsulated (2.80 µg/ml- (25)).

Sample	BMA: MAA	Polymer M_n (Da)	Encapsulated DAPI (µg/ml)
(1)	9: 1	6,900	0.99
(12)	2: 1	7,300	2.00
(17)	4: 1	7,300	0.19
(18)	9: 1	14,100	0.63
(25)	2: 1	7,400	2.80
(26)	9: 1	10,200	0.86

Table 47. Different amounts of DAPI dye were encapsulated in different micelles. The concentration of encapsulated DAPI was calculated using equation $y = 832.18x + 1686.5$, derived from a graph of DAPI concentration versus fluorescence (Figure 60).

Results demonstrated that different amounts of DAPI were encapsulated by different micelles. It should be noted that micelles which encapsulated high levels of DAPI had a higher level of MAA (methacrylic acid) in the core of the micelle (2: 1 BMA: MAA as opposed to 4: 1 or 9: 1). This result corresponds with our hypothesis that the acidic functionality of the micelle core stabilises the encapsulation of basic drugs or dyes. The MAA also increases the polarity of the micelle core and the potential for hydrogen bonding with the encapsulated cargo.

4.2.12 Endocytosis of DAPI encapsulated particles

DAPI-encapsulated micelles successfully delivered DAPI to macrophages. DAPI was also delivered to non-macrophages. Cells were incubated with DAPI-encapsulated micelles for 30 min at 37 °C. Micelles were used at a concentration of 17 µg/ml and cells were used at a concentration of 500,000 cells/ml. The amount of DAPI delivered to cells by different micelles is shown in **Table 48** and **Figure 61**. Different micelles delivered different amounts of DAPI to cells as both the micelle uptake and the amount of DAPI encapsulated varied between different micelles. **(25)** was the optimum micelle for drug delivery as it delivered the most DAPI. This was due to the fact it was able to encapsulate the most DAPI and had a high level of endocytosis. The uptake trend for the micelles displayed in **Table 48** was **(25) > (12) > (1) > (17) > (26) ≈ (18)** for peritoneal macrophages. The amount of DAPI delivered by micelles followed a similar trend: **(25) > (12) > (1) ≈ (17) > (26) ≈ (18)**. These results suggest that, within the ranges tested, the number of micelles endocytosed had a bigger influence on the overall quantity of DAPI delivered than the amount of DAPI encapsulated within each micelle (**(25) > (12) > (1) ≈ (26) > (18) > (17)**). **(25)** delivered the highest quantity of DAPI to peritoneal macrophages and is therefore believed to be a promising candidate for drug and dye delivery. However, micelle **(25)** had low solubility, preventing MALS analysis from being performed. Therefore, the number of polymer chains per particle and the micelle fluorescence could not be calculated for micelle **(25)**. Micelle **(12)** is also a promising option for drug delivery as it delivered the second highest amount of DAPI to cells and is more soluble than micelle **(25)**. The combination of the solubility and high levels of uptake of **(12)** and its ability to encapsulate a large quantity of dye meant it has a significant advantage over other micelles.

Sample	PERI macrophage DAPI MCF	BM macrophage DAPI MCF	BM non-macrophage DAPI MCF
(1)	1,540	300	96.2
(12)	12,900	1,000	658
(17)	1,450	240	93.6
(18)	1,100	193	55.2
(25)	51,300	909	348
(26)	1,040	191	60.4

Table 48. Amount of DAPI delivered to cells by different micelle samples. Amount of DAPI quantified using flow cytometry MCF values. Data shown for peritoneal (PERI) macrophages, bone marrow (BM) macrophages and bone marrow non-macrophages.

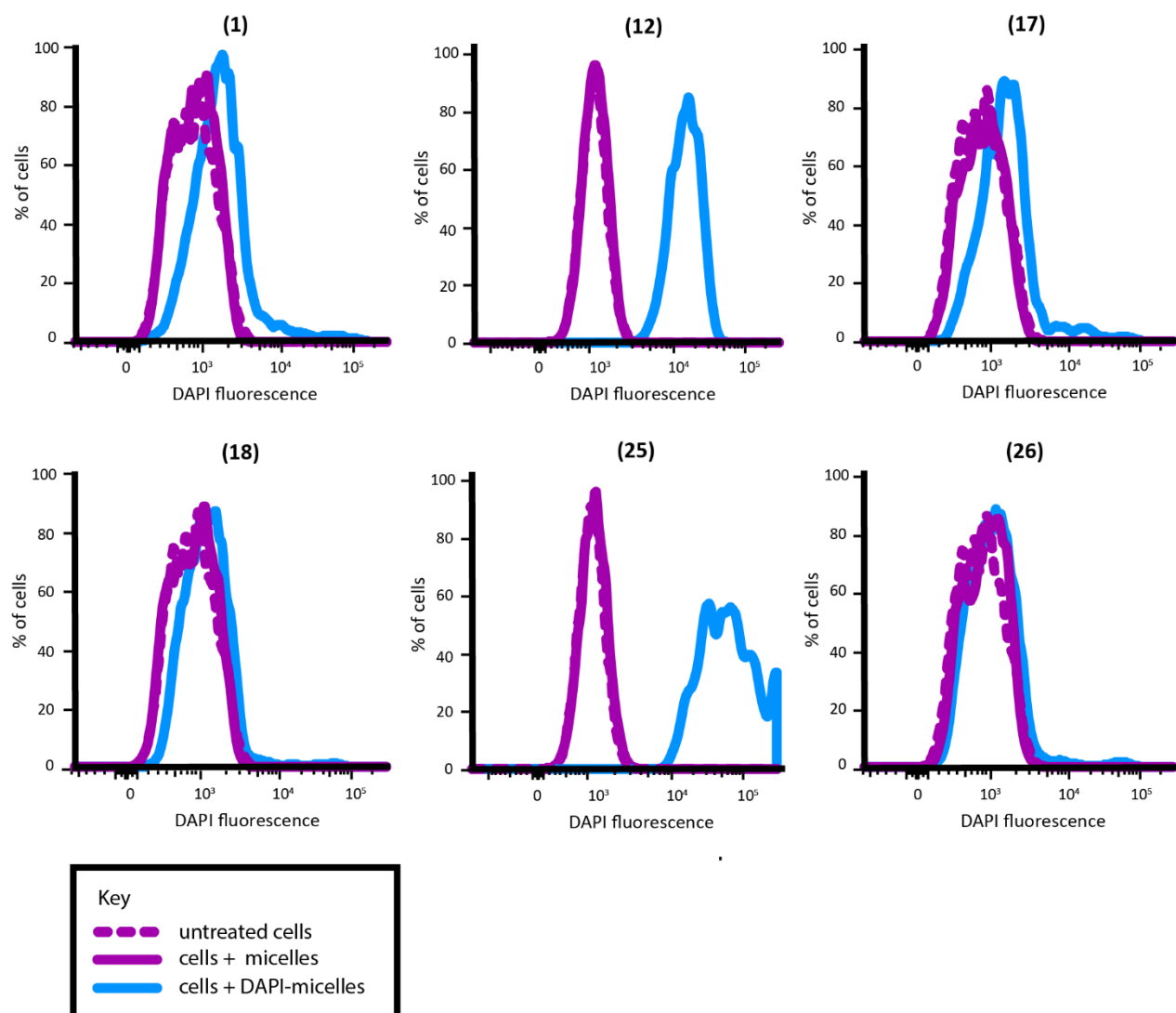


Figure 61. Histograms showing delivery of DAPI to peritoneal macrophages. Untreated cells and micelles not containing DAPI are shown for comparison.

Figure 62 shows the correlation between cellular DAPI fluorescence and rhodamine fluorescence. The linear correlation provides further evidence that micelles are delivering DAPI to cells; cells which endocytosed more micelles contained more DAPI.

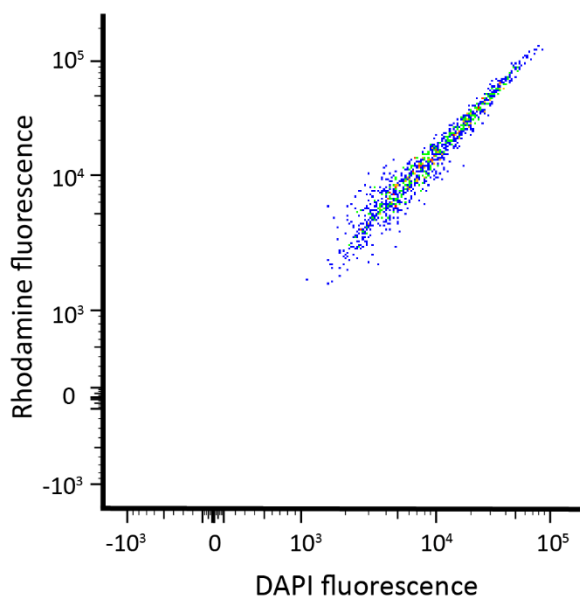


Figure 62. Plot of rhodamine fluorescence against DAPI fluorescence for micelle **(25)** in peritoneal macrophages. There is a linear correlation between the number of micelles endocytosed and the amount of DAPI delivered.

Fluorescence microscopy was also used to determine the presence of DAPI inside cells. DAPI-encapsulated micelles (17 $\mu\text{g/ml}$) were incubated with sorted macrophages (200,000 cells/ml) for 30 min at 37 °C. DAPI fluorescence was easily detected inside the cells due to its high intensity, therefore fluorescence microscopy images were used to further confirm the presence of DAPI inside macrophages.

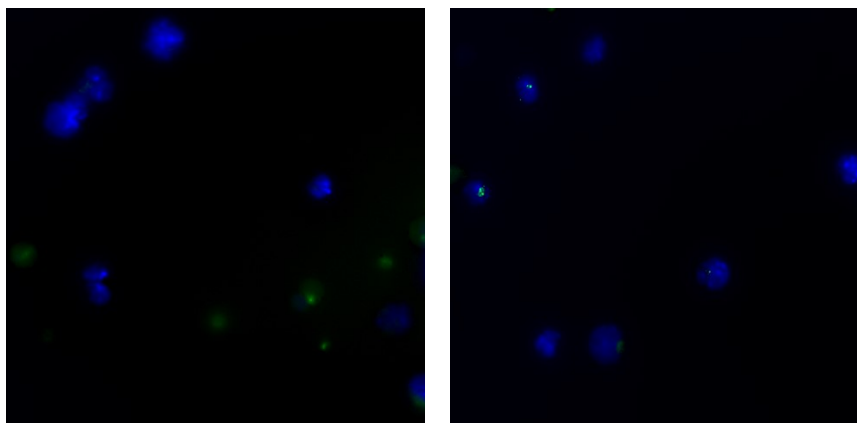


Figure 63. DAPI fluorescence (blue) was detected in large amounts inside cells using fluorescence microscopy.

To confirm the role of micelles in DAPI delivery, free DAPI was incubated with cells at 2.5 $\mu\text{g/ml}$. DAPI fluorescence profiles for DAPI-treated cells were the same as the profiles of untreated cells, no uptake of DAPI was seen when micelles were not used as a delivery system.

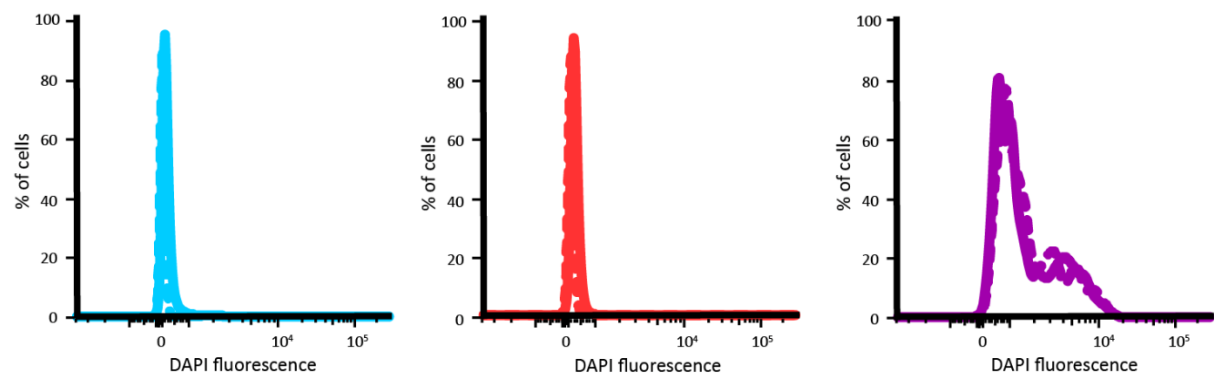


Figure 64. DAPI fluorescence of DAPI treated cells compared to untreated cells (dashed line). Bone marrow non-macrophages (blue), bone marrow macrophages (red) and peritoneal macrophages (purple) were all tested.

4.3 Conclusions

The cellular uptake of a library of fluorescently labelled and crosslinked polymeric micelles was determined. The library of micelles ranged in size from 15 to 170 nm. It was hypothesised that micelles would not be large enough to achieve selective phagocytic uptake but that the size of the particles would have an effect on the level of endocytosis. There was no apparent trend between micelle size and endocytosis within the size range tested but different micelles were internalised different amounts by cells. These results were found to be reproducible and a number of micelles with consistently high levels of endocytosis were identified. Further analysis of micelles of interest was carried out to determine how many polymer chains made up each micelle and this value was used to get a more accurate representation of how many micelles were being endocytosed.

A fluorescent dye, DAPI, was successfully encapsulated in micelles *via* diffusion. It was hypothesised that favourable interactions between the acidic core of the micelle and the basic dye would stabilise the encapsulation. The dye was retained inside the micelles after dialysis. Micelle cores containing the highest ratio of the acidic monomer, MAA (2: 1, BMA: MAA) encapsulated the highest quantity of DAPI (2.8 µg/ml), which supports our hypothesis. Micelle **(12)**, a 46 nm particle assembled from 7,700 Da quasi-block BMA-co-MAA-b-PEGMA-b-NAS-co-MRhB copolymers with a 2: 1 BMA: MAA ratio, has been identified as the most promising for drug delivery, its high uptake alongside its solubility and encapsulation properties meant that it was able to transport large amounts of DAPI into cells.

The main aim of this project, to synthesise polymeric micelles *via* RAFT for delivery to macrophages, has been achieved. In **Chapter II** a library of polymeric micelle particles ranging in size from 15 to 170 nm was synthesised *via* RAFT polymerisation, in **Chapter III** selected polymers were fluorescently labelled using a rhodamine B monomer and a NAS-diamine crosslinking system was used to stabilise the micelles for *in vitro* use. In this chapter cellular studies showed that micelles were successfully internalised by macrophages and that they were able to deliver an encapsulated dye to cells. It should be noted that selective delivery to macrophages has not been achieved as non-macrophage cells also showed high levels of micelle internalisation. This issue of selectivity is the subject of current and future work being carried out in the Fuchter group, by PhD student James Rushworth. The synthesis of larger

micelles is one approach currently being investigated, alongside the decoration of micelles with sugars designed to target macrophage receptors. Although selectivity still needs to be optimised, the micelles synthesised in this project are promising drug delivery vehicles due to their stability at low concentrations, their ability to encapsulate basic cargo and their high level of cellular uptake.

4.4 Experimental

4.4.1 Materials and methods

Reagents, antibodies and solvents were supplied from commercial sources and used as delivered unless otherwise described. Commercial suppliers used were: Sigma Aldrich, Thermofisher Scientific, Becton Dickinson (BD), BioLegend, Life Technologies and Vector Laboratories. The polyamine, JEFFAMINE D230 was kindly donated by HUNTSMAN, an American chemical company.

FACS was performed using an Influx Cell Sorter (BD Biosciences) equipped with 4 lasers: 450 nm, 488 nm, 561 nm and 635 nm. Cells were sorted through at 70 μm nozzle at 25,000 cells/sec. Flow cytometric analysis was performed on a LSR II (BD Biosciences) with 7 solid state lasers: 355 nm, 405 nm, 488 nm, 532 nm, 561 nm, 592 nm and 628 nm. Flowjo X was used to analyse all flow cytometry data.

GPC was performed on a Waters Alliance system equipped with an Alliance 2695 Separations Module (integrated quaternary solvent delivery, solvent degasser and autosampler system), a Waters column heater module, a Waters 2414 RDI refractive index detector, a Waters PDA 2996 photodiode array detector (210 to 400 nm at 1.2 nm) and 4 $\times$ Agilent PL-Gel columns (3 $\times$ PL-Gel Mixed C (5 μm) and 1 $\times$ PL-Gel Mixed E (3 μm) columns), each 300 mm $\times$ 7.8 mm², providing an effective molar mass range of 200 to 2×10^6 . Tetrahydrofuran (THF) high purity solvent (HPLC grade) was pre-filtered through aluminium oxide (90 active neutral, 70–230 mesh) with 0.45 μm filter, and 0.1 g/l 2,6-di-tert-butyl-4-methylphenol (BHT) was added as inhibitor. The filtered THF containing BHT was purged slowly with nitrogen gas and used as an eluent with a flow rate of 1 ml/min at 30 °C. Number (M_n) and weight average (M_w) molar masses were evaluated using Waters Empower-3 software. The GPC columns were calibrated with low dispersity polystyrene (PSt) standards (Polymer Laboratories) ranging from 580 to 7,500,000 g/mol, and molar masses are reported as PSt equivalents. A 3rd-order polynomial was used to fit the $\log M_p$ versus time calibration curve, which was near linear across the molar mass ranges.

The majority of DLS measurements were performed on a Beckman Coulter DelsaNano C Particle Analyzer. BRAND 1.5 ml semi-micro polystyrene (PSt) cuvettes were used. *Measurement parameters*: repetition: 5, temperature: 25 °C, equilibration: 20 sec, wait time: 120 sec, dust limit: 10, upper dust limit: 25, lower dust limit: 50, optimum intensity: 20,000, maximum intensity: 30,000, minimum intensity: 10,000, pinhole: 50 μm . *Analysis parameters*: analysis method: CONTIN, weight function: 1, fitting range: $G_2(\tau)$, $G_2(\tau)_{\text{max}}$: 2, $G_2(\tau)_{\text{min}}$: 1.003. *Cell parameters*: measurement item: size, cell type: size cell, cell centre Z: 4.85 mm, cell centre X: 6.55 mm, correlator type: log, accumulation times: 100. *Dilutant parameters*: dilutant name: water, refractive index: 1.33, viscosity: 0.89 mm^2/s , dielectric constant: 78.3 S/m.

Where specified DLS measurements were performed on a Zetasizer Nano Z-S (Malvern Instruments). BRAND 1.5 ml semi-micro UV cuvettes were used. *Measurement parameters*: repetition: 3, temperature: 25 °C, equilibration: 30 sec, wait time: 120 sec. *Analysis parameters*: analysis method: cumulants. *Cell parameters*: measurement item: size, cell type: low volume disposable sizing cuvette. *Dilutant parameters*: dilutant name: water, refractive index: 1.33, viscosity: 0.89 mm^2/s , dielectric constant: 78.3 S/m.

An EnSpire Multimode Plate Reader was used to measure DAPI and rhodamine fluorescence. Disposable 96 well Nunc DELFIA plates were used. For DAPI measurements, the excitation maximum was 358 nm and the emission maximum was 462 nm. For rhodamine measurements, the excitation maximum was 555 nm and the emission maximum was 575 nm.

Cell washes were performed using PBS 2% fetal bovine serum (PBS-2% FBS) unless otherwise stated. Cells were spun down at a speed of 400 RCF at 8 °C and the supernatant decanted.

4.4.2 Mice

Cells isolated for this work were scavenged from C57Bl/6 mice utilised by the Nilsson group. Mice were bred at Monash Animal Services (Monash University, Clayton, Australia) and ranged in age from 3–12 weeks.

4.4.3 Cell isolation

Isolation of bone marrow cells

The femur, tibia and iliac crest were removed from the hind legs of the mice, cleaned of muscle and fat, then crushed in PBS-2% FBS using a mortar and pestle. The cells were washed with PBS-2% FBS to remove fat and counted using a Sysmex KX-21N automated haematology analyser. Approximately 5×10^7 cells were obtained per leg.

Isolation of peritoneal cells

The outer skin was removed from the area surrounding the peritoneal cavity and PBS-2% FBS (10 ml) was slowly injected into the cavity through a 16-gauge needle. The area was gently massaged to dislodge the maximum number of cells before the cell suspension was removed through the needle. Approximately 2×10^6 cells were obtained per mouse.

4.4.4 Flow cytometry and cell sorting

Live cells for cell sorting and analysis were labelled with antibodies (details in **Table 49**) then suspended in PBS 310 with 2% serum at a concentration of approximately 2×10^7 cells/ml. Cell sorting and analysis gates were set using stained, untreated, bone marrow cells. Prior to cell sorting or analysis, compensations had to be run. Fluorescent dyes have overlapping spectra and to prevent the fluorescence from one dye interfering with the measurement of another, each colour has to be compensated out of the other colours being used in that experiment. The compensations used were: unstained cells, CD45-FITC, CD45-APC-Cy7, CD45-AF647 and CD45-BV510 labelled cells, Pacific Blue labelled $2 \mu\text{m}$ latex beads and rhodamine labelled $1 \mu\text{m}$ latex beads. The endocytosis of micelles was determined by rhodamine fluorescence detected in cells. Rhodamine was excited with the 561 nm laser and fluorescence emission was detected with a 564–606 nm band pass filter. The successful delivery of DAPI to cells was determined by DAPI fluorescence in cells. DAPI was excited with the 355 nm laser and detected with a 425–475 nm band pass filter.

Antibody labelling

Cells were immunolabelled for cell sorting and flow cytometry with the following antibodies: Gr-1-FITC, CD11b-APC-Cy7 and F4/80-AF647. When fluorescence microscopy was used, cells

were also labelled with CD45-BV510 to help make cells easier to identify. Antibody details for cell sorting and analysis are displayed in **Table 49**.

Antibody	Description	Clone	Conc. (µg/ml)	Supplier
CD45-FITC	FITC Rat anti-mouse CD45	30-F11	1.0	BD Biosciences
CD45-APC-Cy7	APC-Cy7 Rat anti-mouse CD45	30-F11	1.0	BD Pharmigen
CD45-AF647	Alexa Fluor 647 anti-mouse CD45	30-F11	0.25	Biolegend
CD45-BV510	BV510 Rat anti-mouse CD45	30-F11	1.0	BD Horizon
Gr-1-FITC	FITC anti-mouse Ly-6G and Ly-6C (Gr-1)	RB6-8C5	0.25	BD Pharmigen
CD11b-APC-Cy7	APC-Cy7 Rat anti-mouse CD11b	M1/70	0.50	BD Pharmigen
F4/80-AF647	Alexa Fluor 647 anti-mouse F4/80	BM8	10	Biolegend

Table 49. Details of antibodies used for cell sorting and analysis. Concentration displayed is the final concentration used.

Sorting strategy

Sorted populations of macrophages (Gr-1-FITC⁻, Mac-1-APC-Cy7⁺, F4/80-AF647⁺ cells) and non-macrophages (Gr-1-FITC⁻, Mac-1-APC-Cy7⁻, F4/80-AF647⁻ cells) were collected.²⁶⁰ It should be noted that peritoneal macrophages were GR-1-FITC^{low} opposed to GR-1-FITC⁻; activated macrophages are known to be Gr-1⁺.^{261,262} Peritoneal macrophages also had much higher levels of F4/80 than bone marrow macrophages.^{250,263} In **Gate 1 (Figure 65)** single cells were selected. Cell height (FSC-H) versus cell area (FSC-A) was plotted, and any events which did not fit the correlation between area and height were excluded; cell doublets or multiplets were excluded from this gate. **Gate 2** was sequentially gated from **Gate 1** and isolated nucleated cells from cell debris and non-nucleated cells (e.g. red blood cells) in a plot of side scatter versus forward scatter. Forward scatter is the scattering of light by the laser in the forwards direction and depends on the volume of the cell. Side scatter is light scattered at different angles and is caused by the size and contents of the cell, more complex cells scatter more light.^{264,265} **Gates 3 and 4** were sequentially applied to a plot of MAC-1, versus Gr-1. **Gate 3** isolated Mac-1⁺, Gr-1⁻ cells, while **Gate 4** isolated Mac-1⁻, Gr-1⁻ cells. **Gate 5** was sequentially gated from **Gate 3** and isolated Mac-1⁺, F4/80⁺ cells (macrophages). **Gate 6** was sequentially gated from **Gate 4** to exclude F4/80⁺ cells from the non-macrophage population.

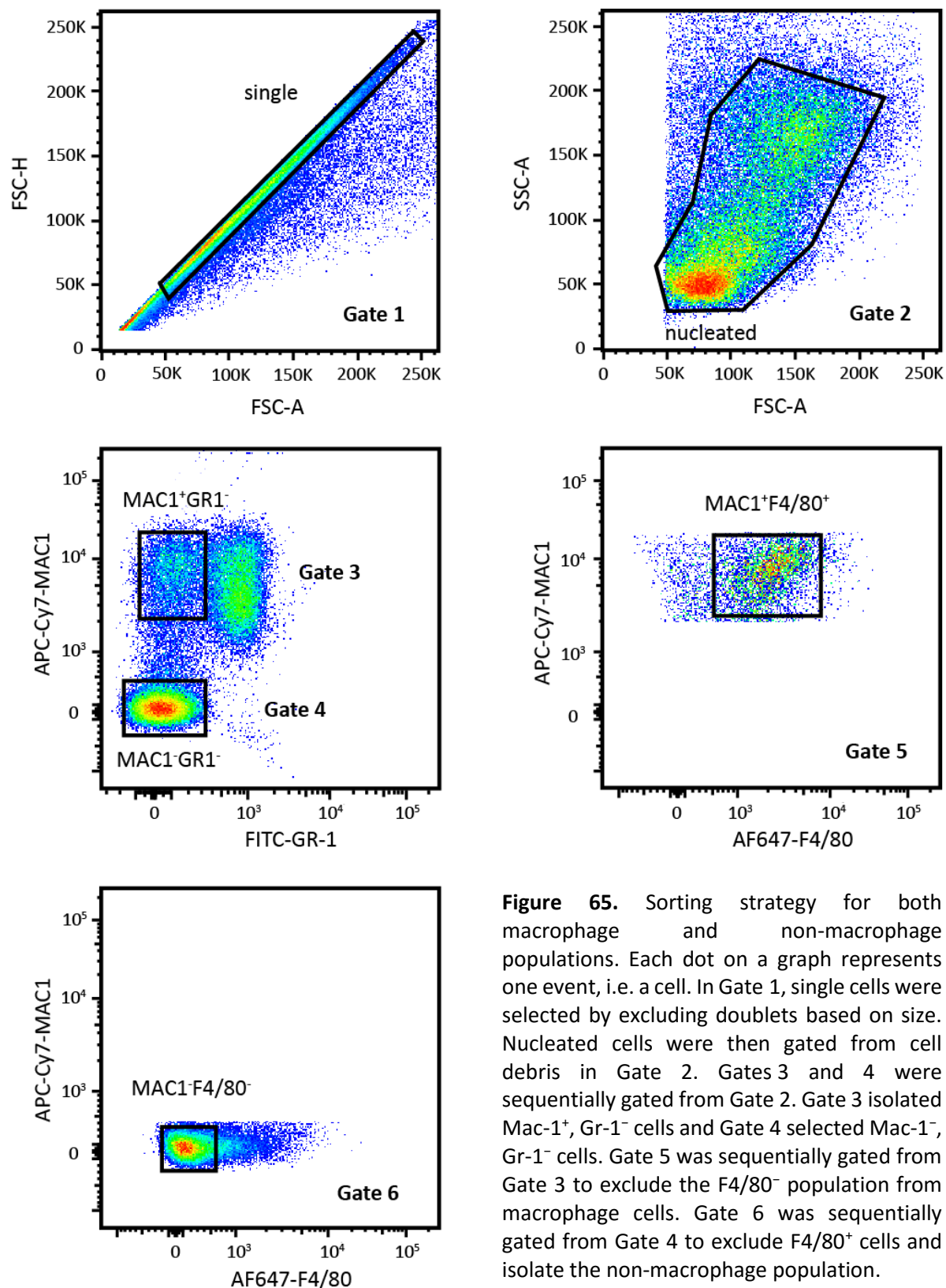


Figure 65. Sorting strategy for both macrophage and non-macrophage populations. Each dot on a graph represents one event, i.e. a cell. In Gate 1, single cells were selected by excluding doublets based on size. Nucleated cells were then gated from cell debris in Gate 2. Gates 3 and 4 were sequentially gated from Gate 2. Gate 3 isolated Mac-1⁺, Gr-1⁻ cells and Gate 4 selected Mac-1⁻, Gr-1⁻ cells. Gate 5 was sequentially gated from Gate 3 to exclude the F4/80⁺ population from macrophage cells. Gate 6 was sequentially gated from Gate 4 to exclude F4/80⁺ cells and isolate the non-macrophage population.

4.4.5 General procedure for cell uptake studies

Sorted cells

Post sorting, cells were counted manually using an Optik Labour Neubauer Improved Bright Line haemocytometer. Cell populations were subsequently resuspended at 2×10^5 cells/ml in PBS-2% FBS and 200,000 cells aliquoted per test condition. Solutions of micelles in PBS 0.5% bovine serum albumin (PBS 0.5% BSA) (200 μ l, 100 μ g/ml) were added to the cell suspensions (giving final micelle concentrations of 17 μ g/ml) with the exception of positive and negative controls. The positive control was 2 μ m Pacific Blue latex beads, previously shown to be phagocytosed by macrophages but not endocytosed other cell types. In negative controls PBS 0.5% BSA was added to cells. All cells were incubated for 30 min at 37 °C. Following incubation, cells were washed and resuspended in 0.1 μ g/ml propidium iodide (PI) in PBS 0.5% BSA at a concentration of approximately 1×10^6 cells/ml.

Unsorted cells

After immunolabelling, cells were suspended at a concentration of 200,000 cells/ml in PBS-2% FBS and 500,000 cells were aliquoted per test condition. Solutions of micelle particles dissolved in PBS 0.5% BSA (500 μ l, 100 μ g/ml) were added to the cell suspensions (giving final micelle concentrations of 17 μ g/ml) with the exception of positive and negative controls. Controls were the same as described above for sorted cells. All cells were incubated for 30 min at 37 °C. Following incubation, cells were washed and resuspended in 0.1 μ g/ml PI in PBS 0.5% BSA at a concentration of approximately 1×10^6 cells/ml.

4.4.6 Controls

Figure 66 is an overlay of histograms from positive and negative controls. The cells were primary macrophages and non-macrophages harvested from murine bone marrow. Peak 1 represents non-macrophages, which have not phagocytosed the Pacific Blue latex beads. Peak 2 represents the macrophages which have not phagocytosed the Pacific Blue latex beads and peak 3 represents macrophages which have phagocytosed Pacific Blue beads. Pacific Blue latex beads were phagocytosed by 27.9% of the macrophages and 0% of non-macrophages.

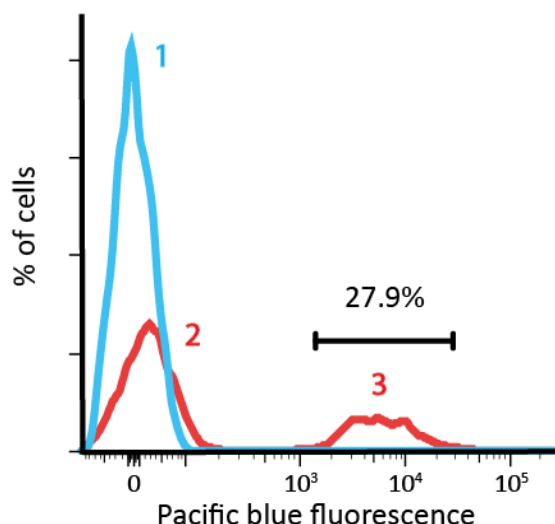


Figure 66. Positive control: Flow cytometric analysis of phagocytosed Pacific Blue beads (2 μm) by macrophages (red) and non-macrophages (blue) following phagocytosis assay. Cells that did not phagocytose beads, peaks 1 and 2, lie to the left of the histogram, as they are non-fluorescent. Cells that phagocytosed latex beads are fluorescent and lie to the right (3).

4.4.7 Fluorescence microscopy

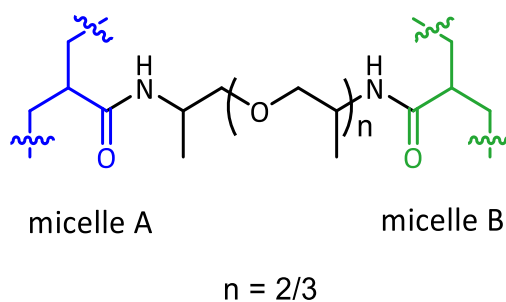
All fluorescence microscopy images were acquired using an Olympus BX51 microscope. After FACS analysis, cells were transferred to 2 ml Eppendorf tubes, spun down, dry pelleted and resuspended in 5 μl of VECTASHIELD Antifade Mounting Medium (Vector Laboratories). Cell suspensions were transferred to microscope slides (Thermo Scientific Superfrost) with 20 mm x 20 mm glass coverslips.

4.4.8 Labelling latex beads

The protocol described was used to label 60 nm, 400 nm and 1 μm latex beads with rhodamine B. Aliphatic amine latex beads, 2% w/v 0.06 μm , 0.4 μm and 1.0 μm were purchased from Life Technologies (Thermo Fisher Scientific).

A slurry of latex beads (1 ml, 2% w/v) was equilibrated in a 0.1 M NaHCO_3 bicarbonate buffer (pH 8.32). The beads were washed and pelleted *via* centrifugation (3 x 5 min at rcf: 20,817). After the final wash, beads were pelleted and made up to a volume of 200 μl of NaHCO_3 to give a 10% w/v slurry. 10% w/v slurry (100 μl) was added to a 1.5 ml Eppendorf containing Rh-NHS dye, 5(6)-carboxytetramethylrhodamine *N*-succinimide, (1 mg, 2 μmol). The mixture was vortexed for approx. 30 sec and then mixed for 10 min. Beads were purified using dialysis, using cassettes, 'Slide-A-Lyzer Dialysis' purchased from Thermo Scientific, 3,500 MWCO, 0.5–3 ml capacity, product # 66330.

4.4.9 Inter-micelle crosslinking



Polymer was dissolved in deionised water at a range of concentrations (see **Table 50** for concentrations and quantities), due to the low quantities of JEFFAMINE required, larger quantities were weighed out and diluted. The solution was placed under a blanket of nitrogen before the polyetheramine, JEFFAMINE D230, was added. The reaction was stirred at room temperature for 20 h. Solvent was removed under reduced pressure. DLS analysis was used to determine the diameter of particles after crosslinking.

Micelle	Polymer	JEFFAMINE	WATER	Polymer conc. (mg/ml)
(14)	102 mg, 7.0 μ mol	2.3 mg, 10 μ mol	500 ml	0.2
(14)	13 mg, 0.9 μ mol	0.3 mg, 1.3 μ mol	30 ml	0.4
(14)	13 mg, 0.9 μ mol	0.3 mg, 1.3 μ mol	16 ml	0.8

Table 50. Quantities of polymer, JEFFAMINE and water and polymer concentrations used in inter-micelle crosslinking study.

4.4.10 Encapsulation of DAPI

Crosslinked micelle particles (3 mg) were dissolved in water (2.3 ml) and 4',6-diamidino-2-phenylindole solution (DAPI) (720 μ l, 5 mg/ml) was added. Reaction mixture was vortexed for 1 min and then stirred for 24 h at room temperature in the absence of light. Micelle particles were purified *via* dialysis in water for 48 h. Dialysis was initially performed in PBS, MW 310, as cellular studies require that the micelles were dissolved in PBS. However, dialysis using PBS caused the formation of a pale yellow precipitate (believed to be the product) to form in the dialysis cartridge. For this reason, dialysis was performed in water and micelles were only suspended in PBS at significantly lower concentrations (100 μ g/ml).

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APPENDICES

Micelle	Fluorescence (RFU)
(1)	433
(2)	2312
(3)	2447
(4)	1947
(5)	1692
(6)	9031
(7)	3479
(8)	1002
(9)	2197
(10)	754
(11)	2765
(12)	318
(14)	821
(15)	1238
(16)	706
(17)	512
(18)	657
(19)	756
(20)	854
(21)	595
(22)	1335
(23)	806
(24)	784
(25)	715
(26)	2194
(27)	508

Table 51. Sample fluorescence at 250 µg/ml, displayed in relative fluorescence units (RFU). Sample fluorescence was calculated from a concentration vs fluorescence plot of each sample.

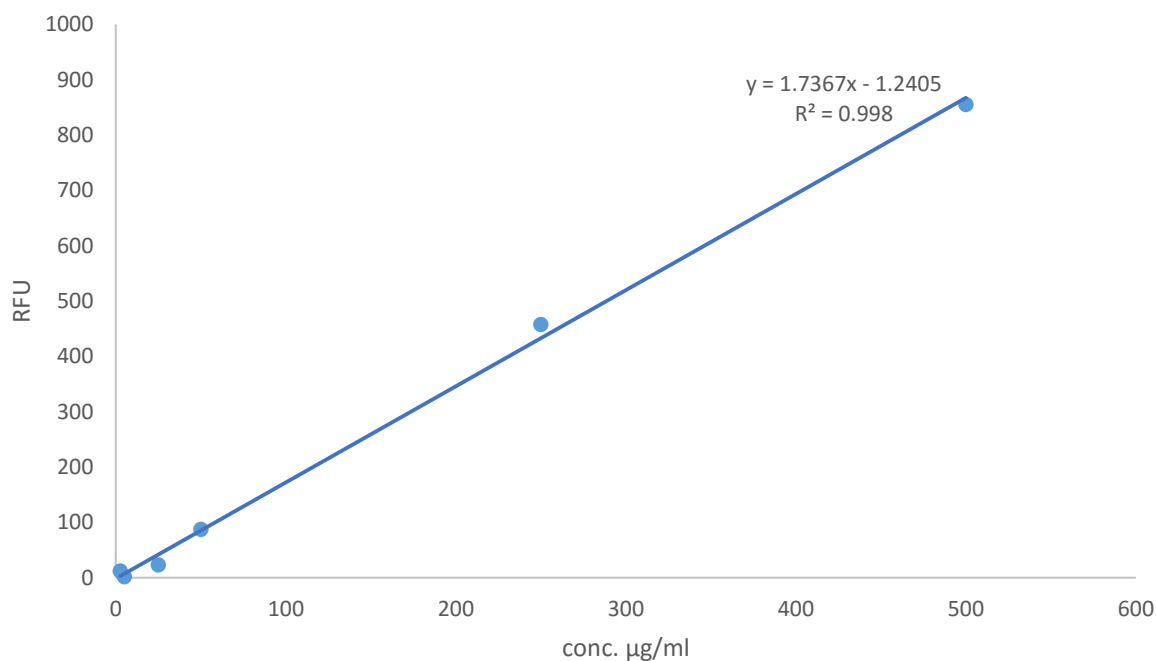


Figure 67. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle (1). The equation derived from the line of best fit ($y = 1.7367x - 1.2405$) was used to calculate the RFU value for a 250 µg/ml solution.

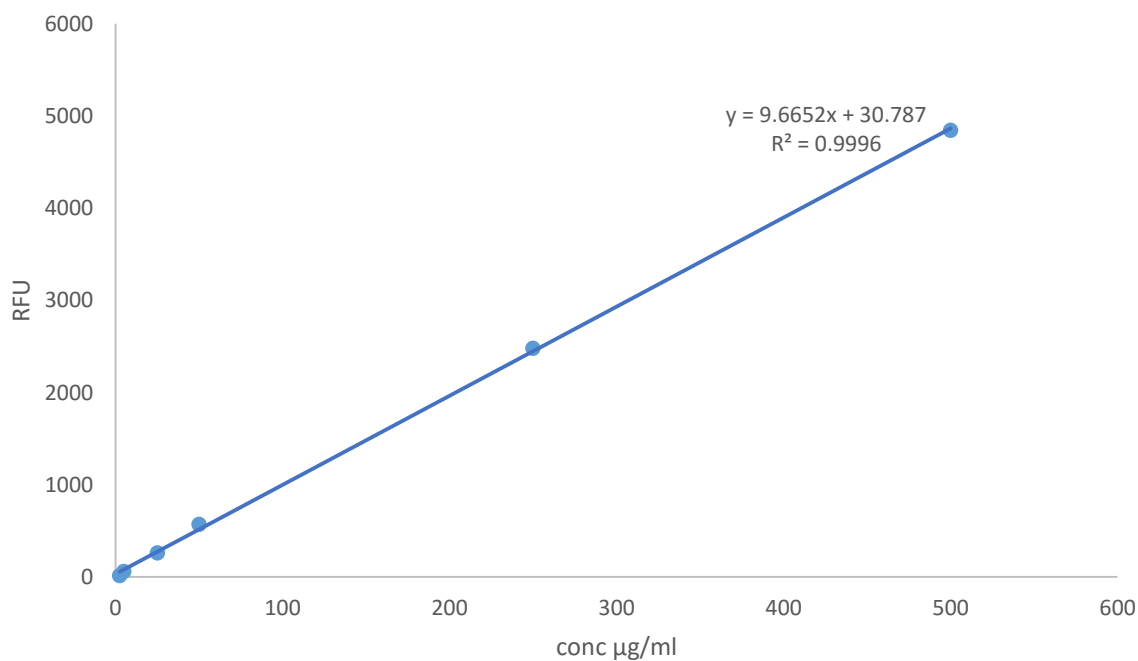


Figure 68. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle (3). The equation derived from the line of best fit ($y = 9.6652x + 30.787$) was used to calculate the RFU value for a 250 µg/ml solution.

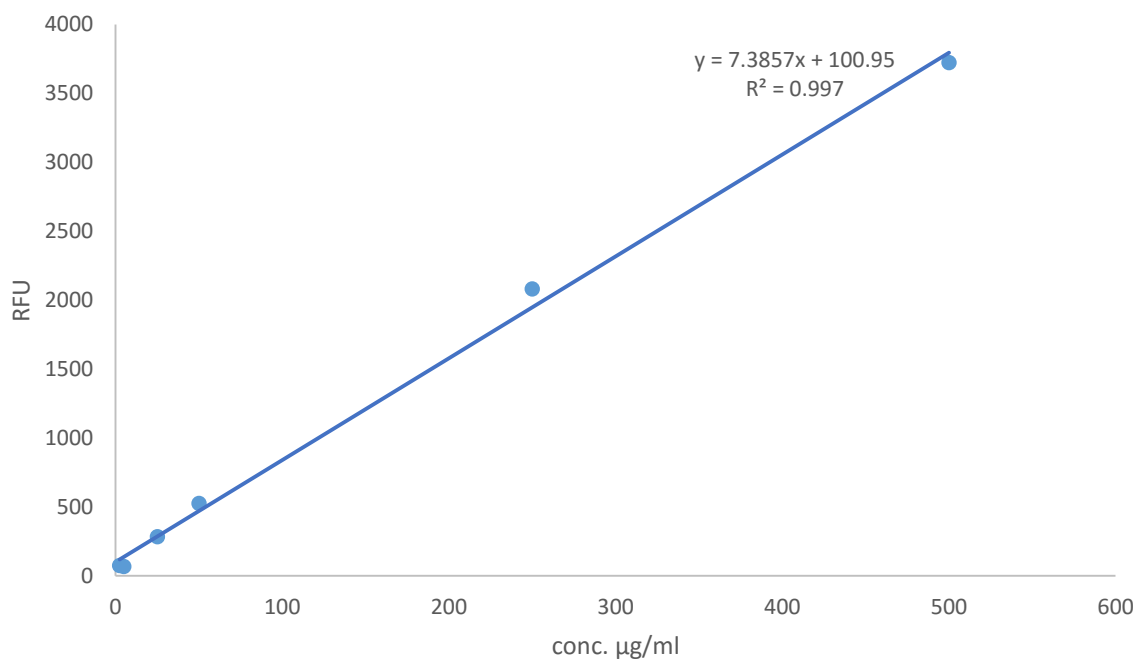


Figure 69. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle **(4)**. The equation derived from the line of best fit ($y = 7.3857x + 100.95$) was used to calculate the RFU value for a 250 µg/ml solution.

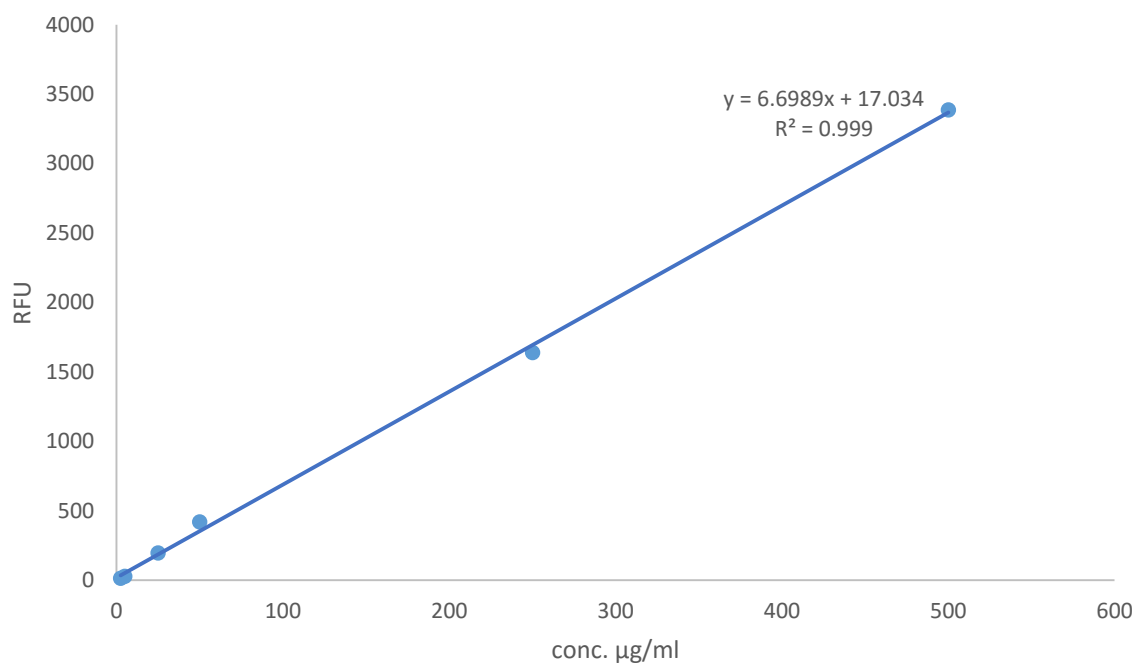


Figure 70. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle **(5)**. The equation derived from the line of best fit ($y = 6.6989x + 17.034$) was used to calculate the RFU value for a 250 µg/ml solution.

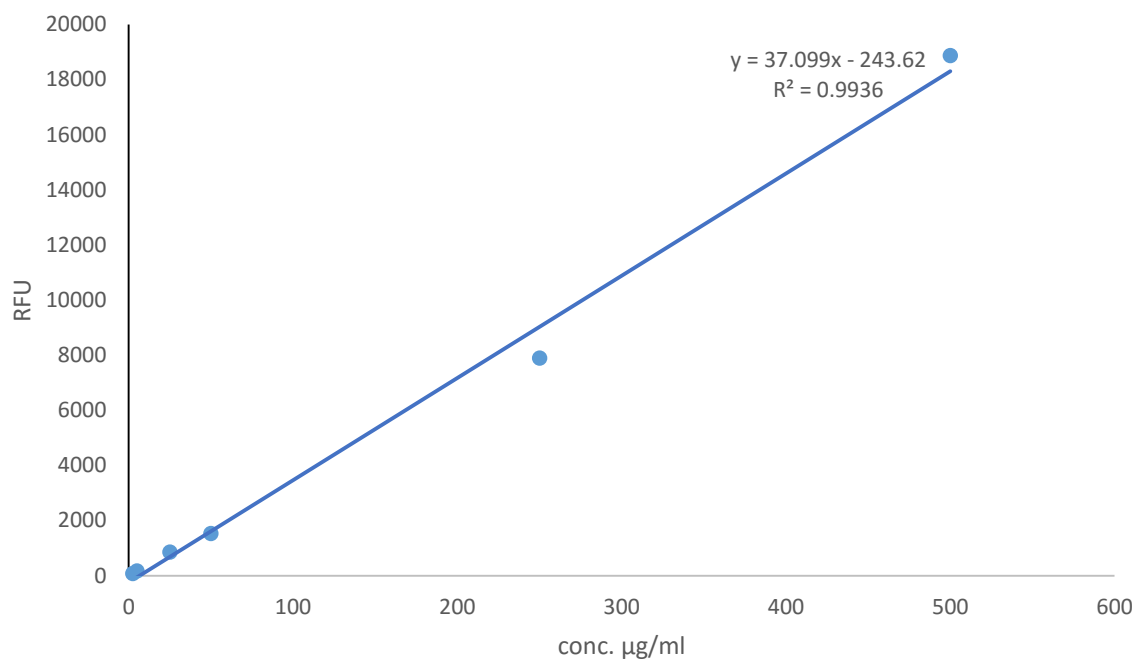


Figure 71. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle **(6)**. The equation derived from the line of best fit ($y = 37.099x - 243.62$) was used to calculate the RFU value for a 250 µg/ml solution.

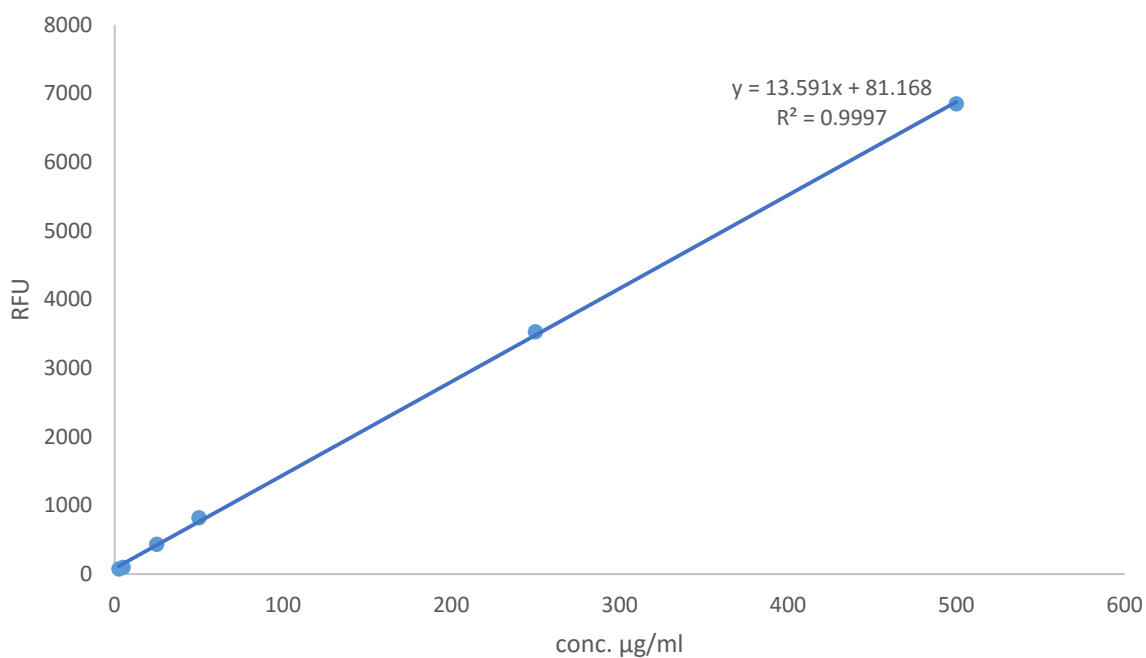


Figure 72. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle **(7)**. The equation derived from the line of best fit ($y = 13.591x + 81.168$) was used to calculate the RFU value for a 250 µg/ml solution.

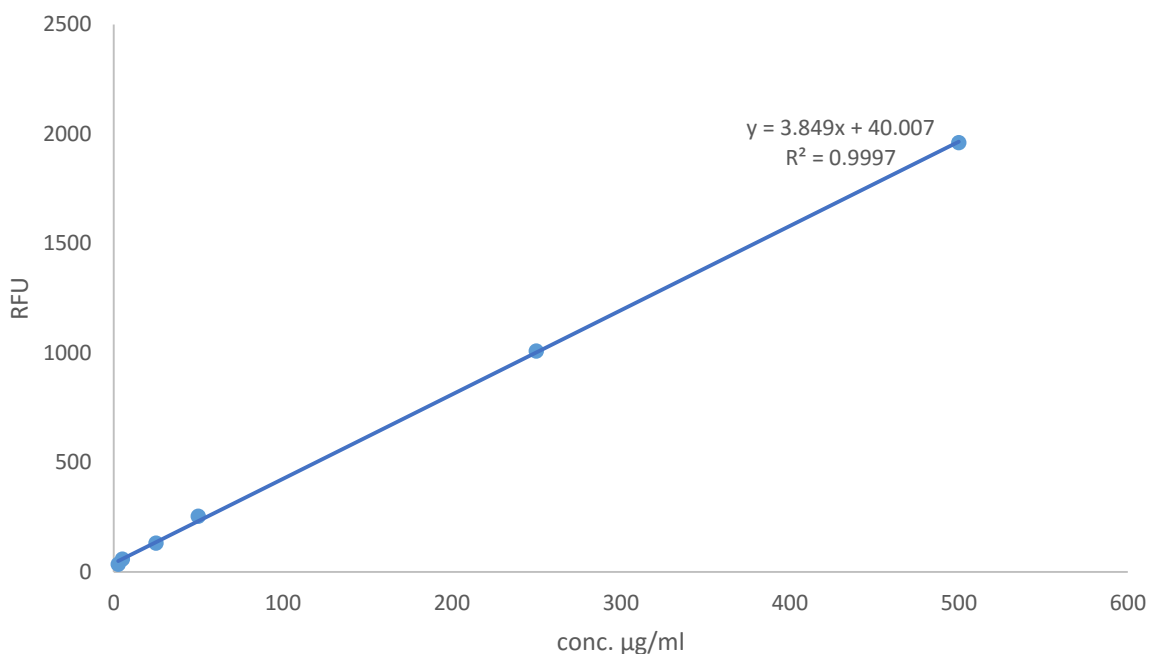


Figure 73. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle (8). The equation derived from the line of best fit ($y = 3.849x + 40.007$) was used to calculate the RFU value for a 250 µg/ml solution.

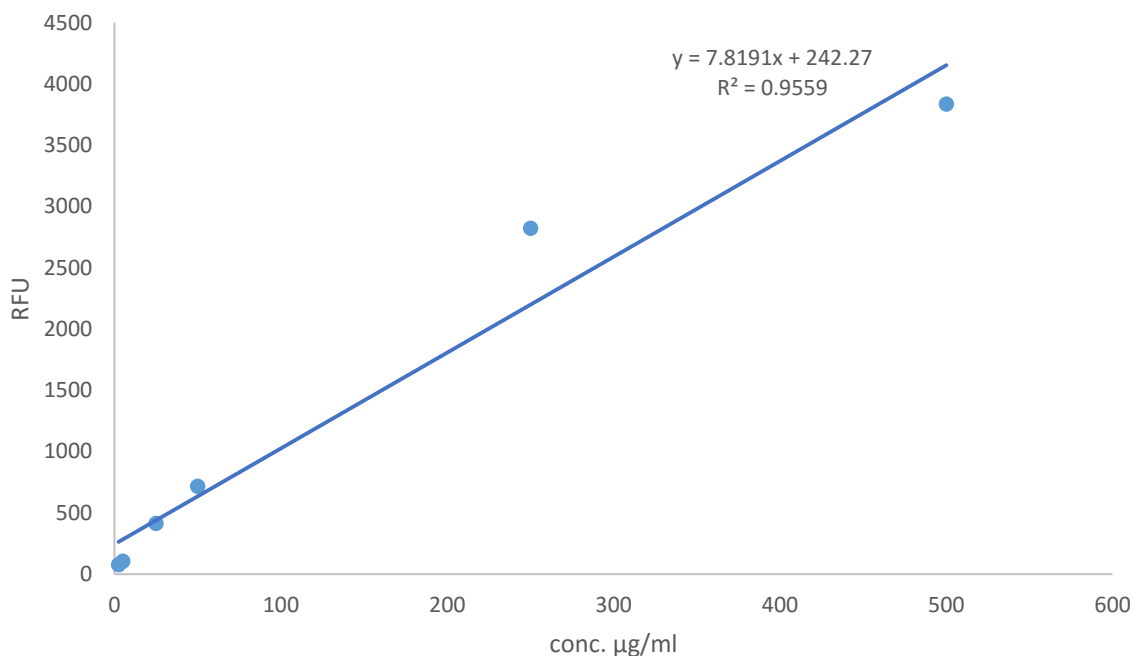


Figure 74. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle (9). The equation derived from the line of best fit ($y = 7.8191x + 242.27$) was used to calculate the RFU value for a 250 µg/ml solution.

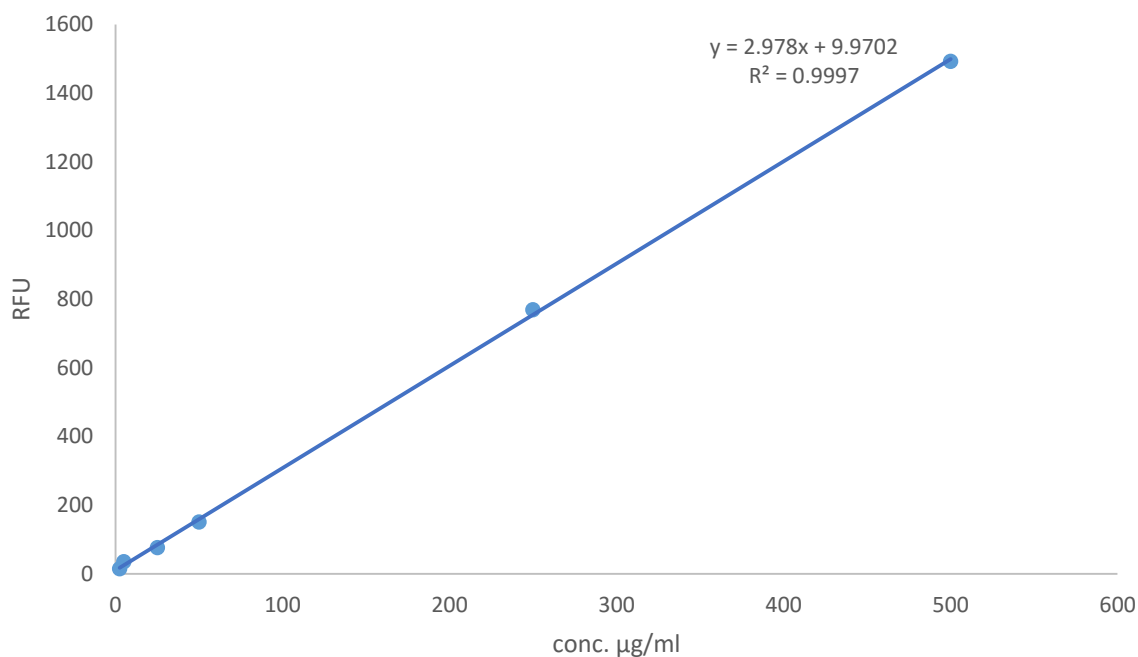


Figure 75. Plot of relative fluorescence units (RFU) versus concentration ($\mu\text{g/ml}$) for micelle **(10)**. The equation derived from the line of best fit ($y = 2.978x + 9.9702$) was used to calculate the RFU value for a 250 $\mu\text{g/ml}$ solution.

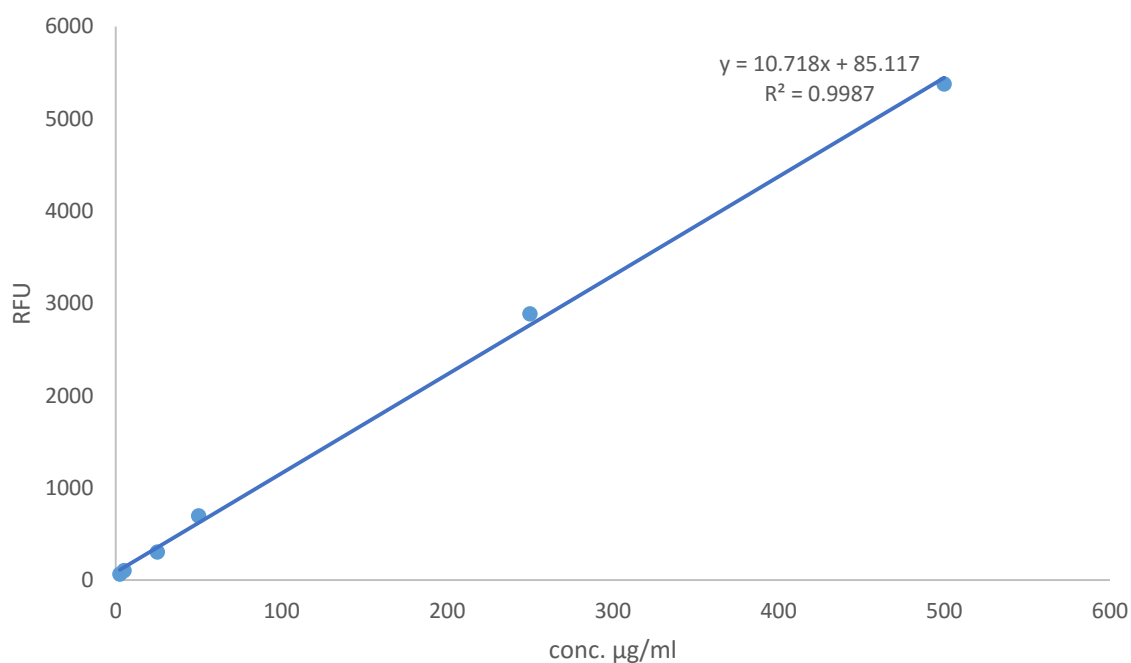


Figure 76. Plot of relative fluorescence units (RFU) versus concentration ($\mu\text{g/ml}$) for micelle **(11)**. The equation derived from the line of best fit ($y = 10.718x + 85.117$) was used to calculate the RFU value for a 250 $\mu\text{g/ml}$ solution.

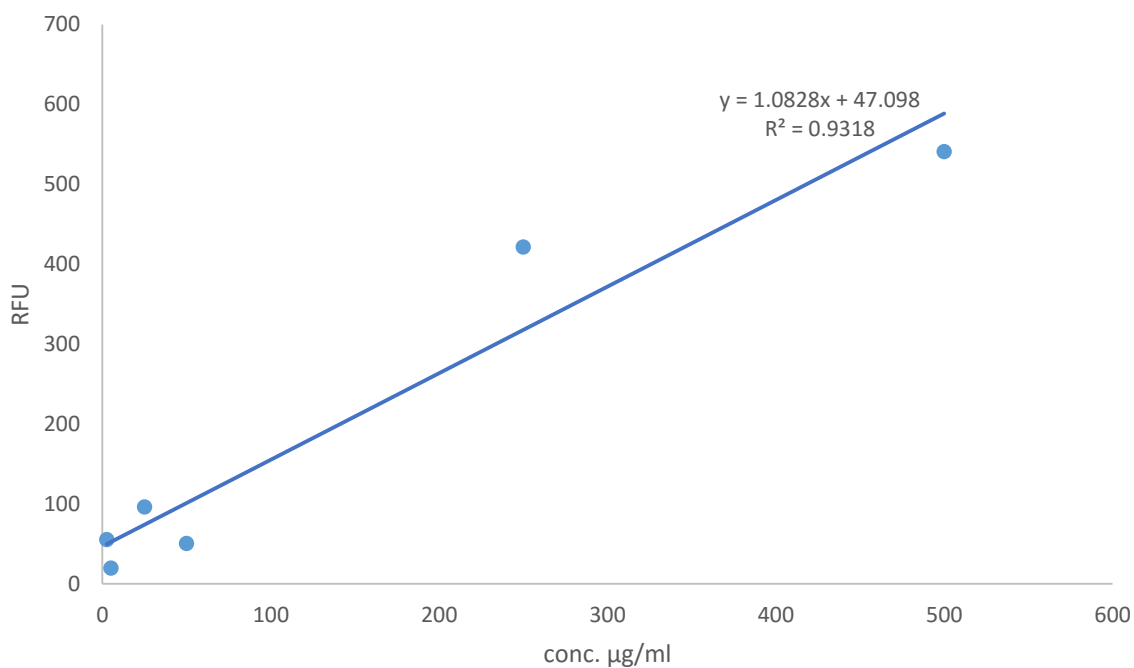


Figure 77. Plot of relative fluorescence units (RFU) versus concentration ($\mu\text{g/ml}$) for micelle **(12)**. The equation derived from the line of best fit ($y = 1.0828x + 47.098$) was used to calculate the RFU value for a 250 $\mu\text{g/ml}$ solution.

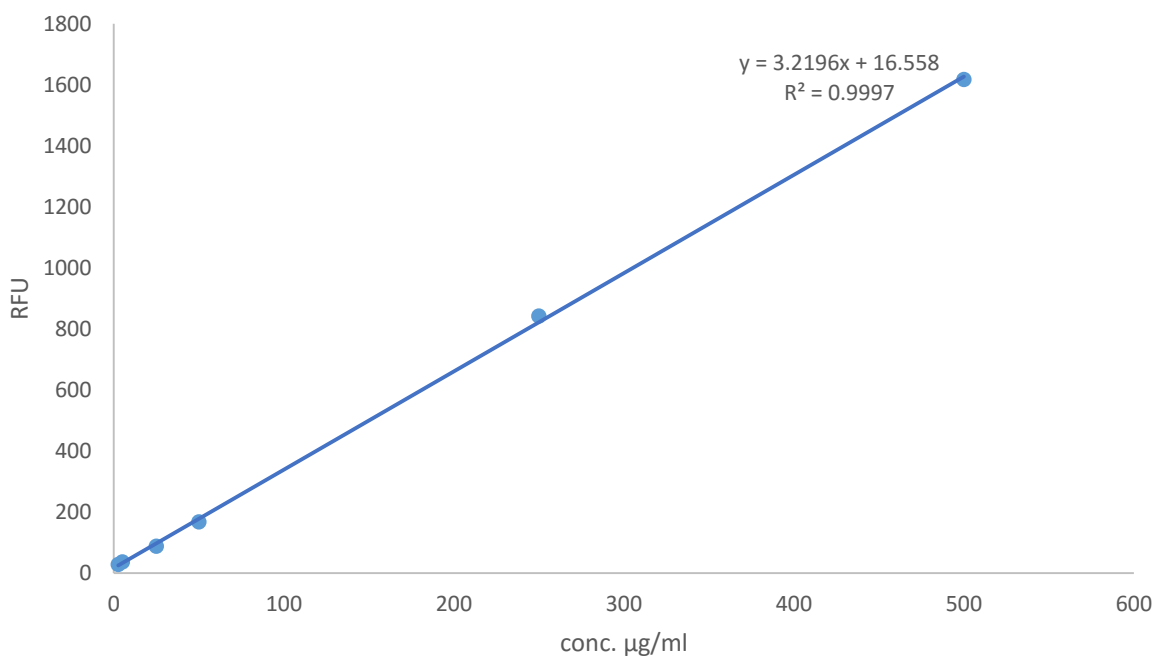


Figure 78. Plot of relative fluorescence units (RFU) versus concentration ($\mu\text{g/ml}$) for micelle **(14)**. The equation derived from the line of best fit ($y = 3.2196x + 16.558$) was used to calculate the RFU value for a 250 $\mu\text{g/ml}$ solution.

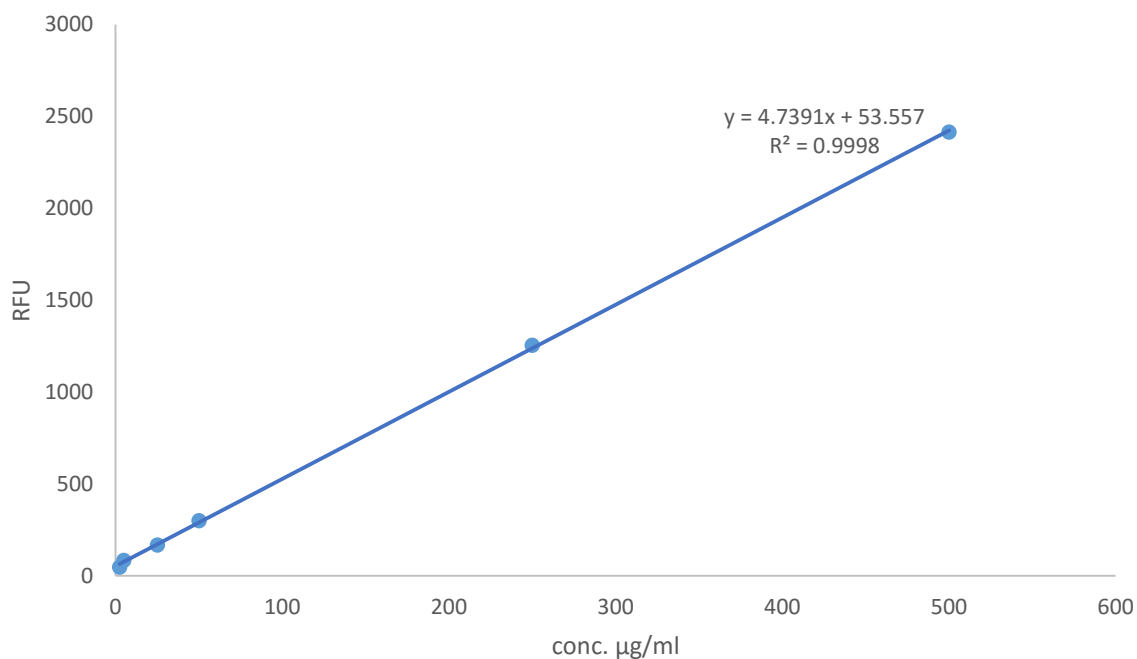


Figure 79. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle **(15)**. The equation derived from the line of best fit ($y = 4.7391x + 53.557$) was used to calculate the RFU value for a 250 µg/ml solution.

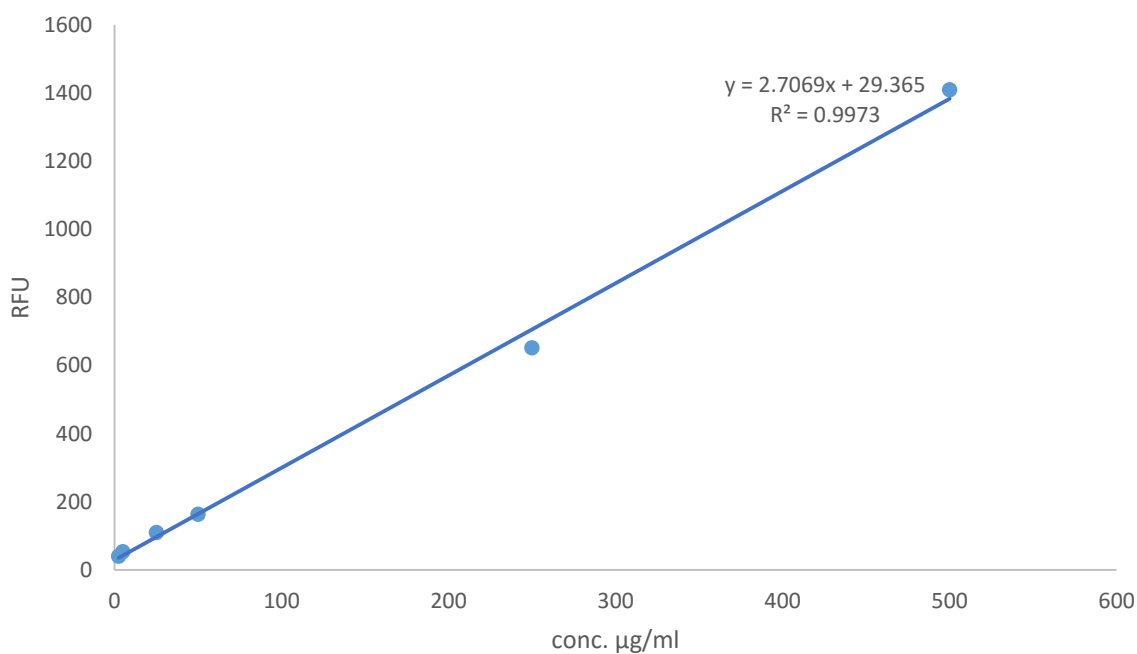


Figure 80. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle **(16)**. The equation derived from the line of best fit ($y = 2.7069x + 29.365$) was used to calculate the RFU value for a 250 µg/ml solution.

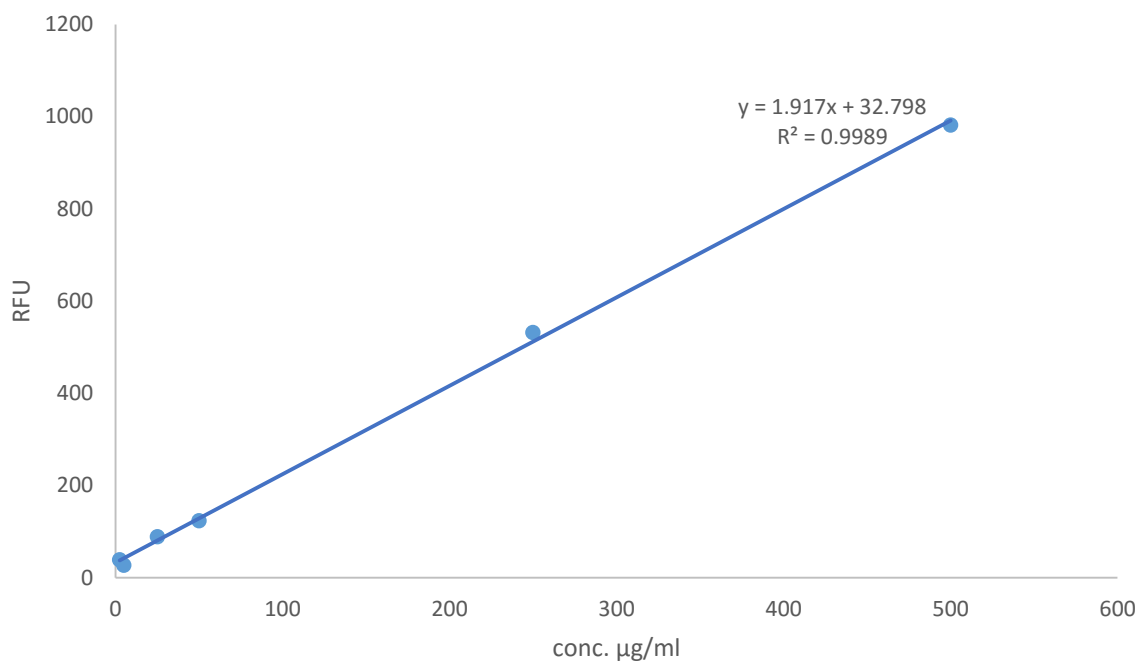


Figure 81. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle (17). The equation derived from the line of best fit ($y = 1.917x + 32.798$) was used to calculate the RFU value for a 250 µg/ml solution.

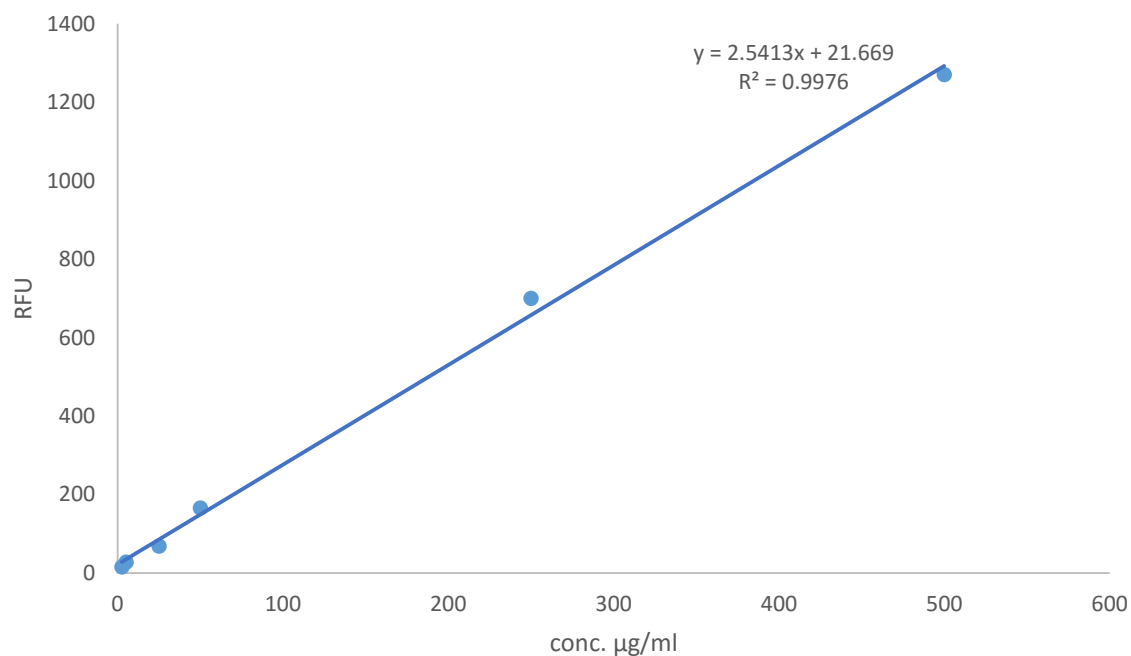


Figure 82. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle (18). The equation derived from the line of best fit ($y = 2.5413x + 21.669$) was used to calculate the RFU value for a 250 µg/ml solution.

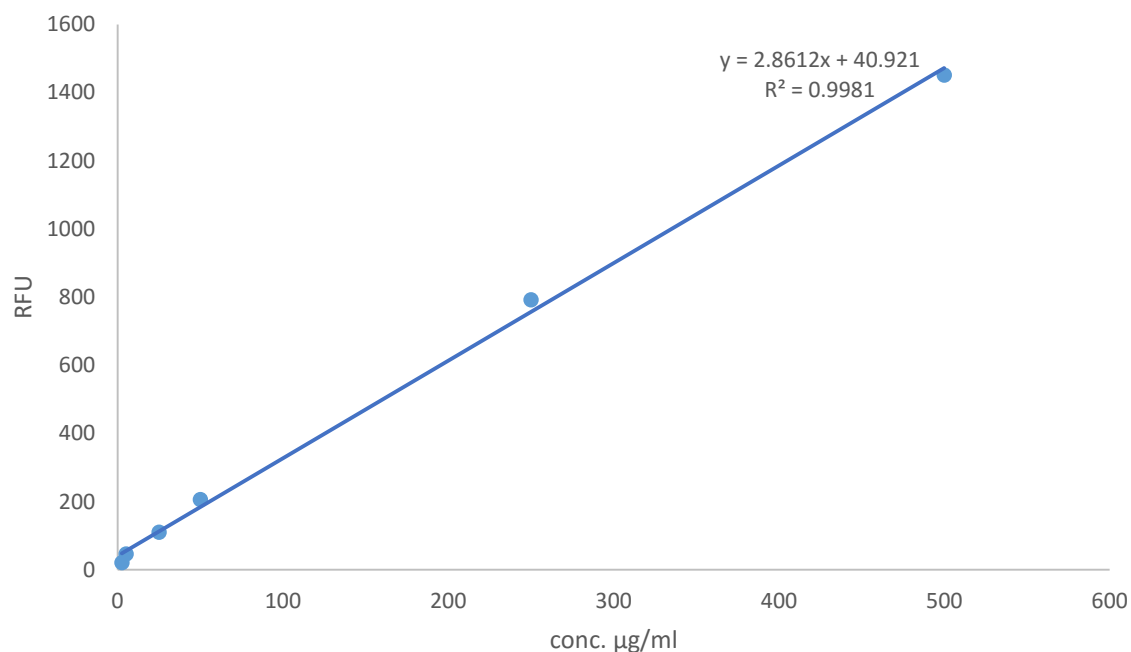


Figure 83. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle **(19)**. The equation derived from the line of best fit ($y = 2.8612x + 40.921$) was used to calculate the RFU value for a 250 µg/ml solution.

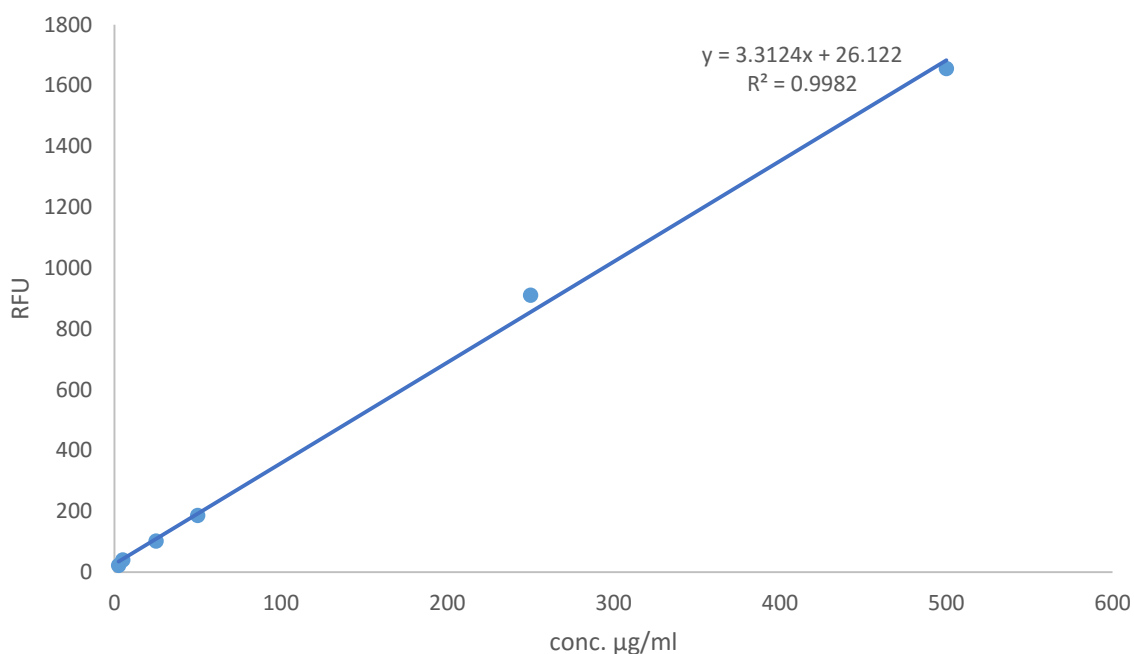


Figure 84. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle **(20)**. The equation derived from the line of best fit ($y = 3.3124x + 26.122$) was used to calculate the RFU value for a 250 µg/ml solution.

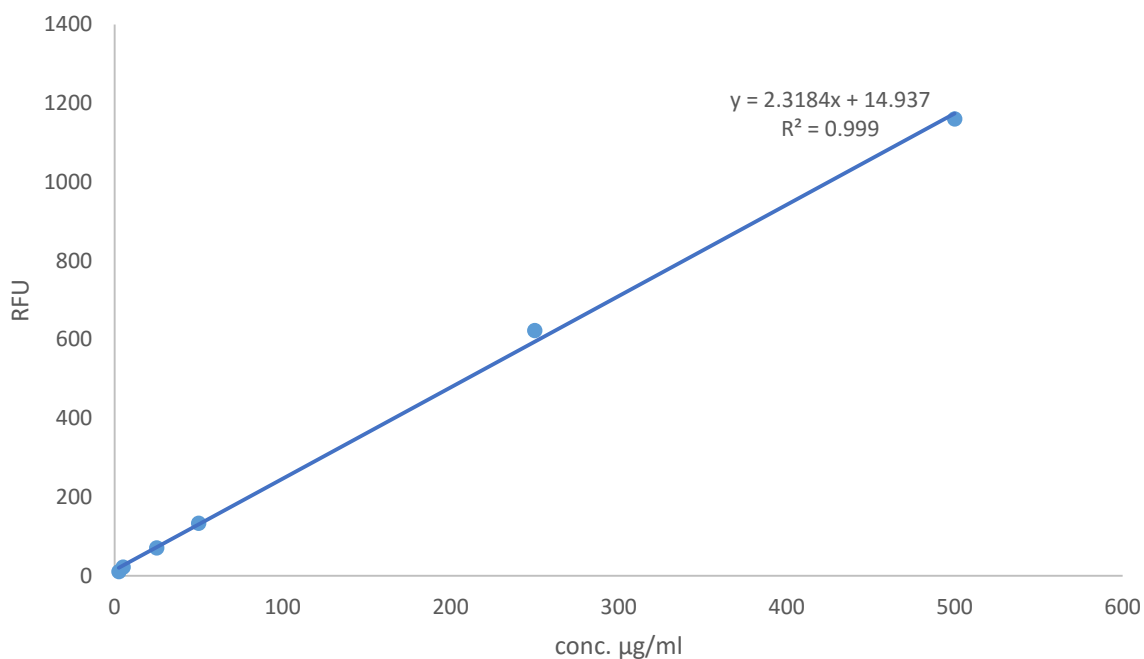


Figure 85. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle **(21)**. The equation derived from the line of best fit ($y = 2.3184x + 14.937$) was used to calculate the RFU value for a 250 µg/ml solution.

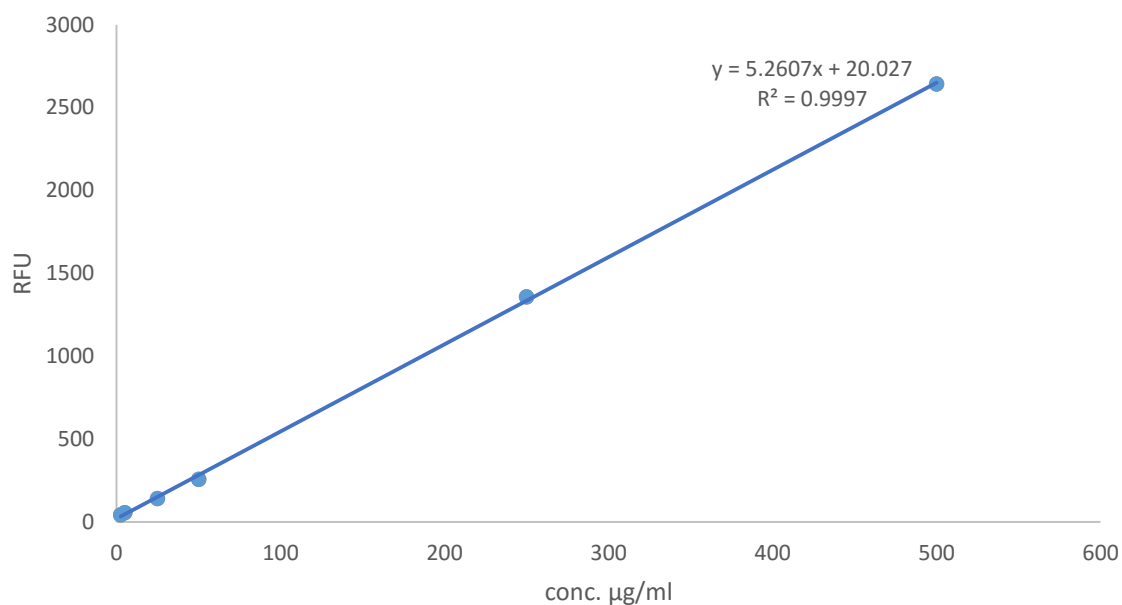


Figure 86. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle **(22)**. The equation derived from the line of best fit ($y = 5.2607x + 20.027$) was used to calculate the RFU value for a 250 µg/ml solution.

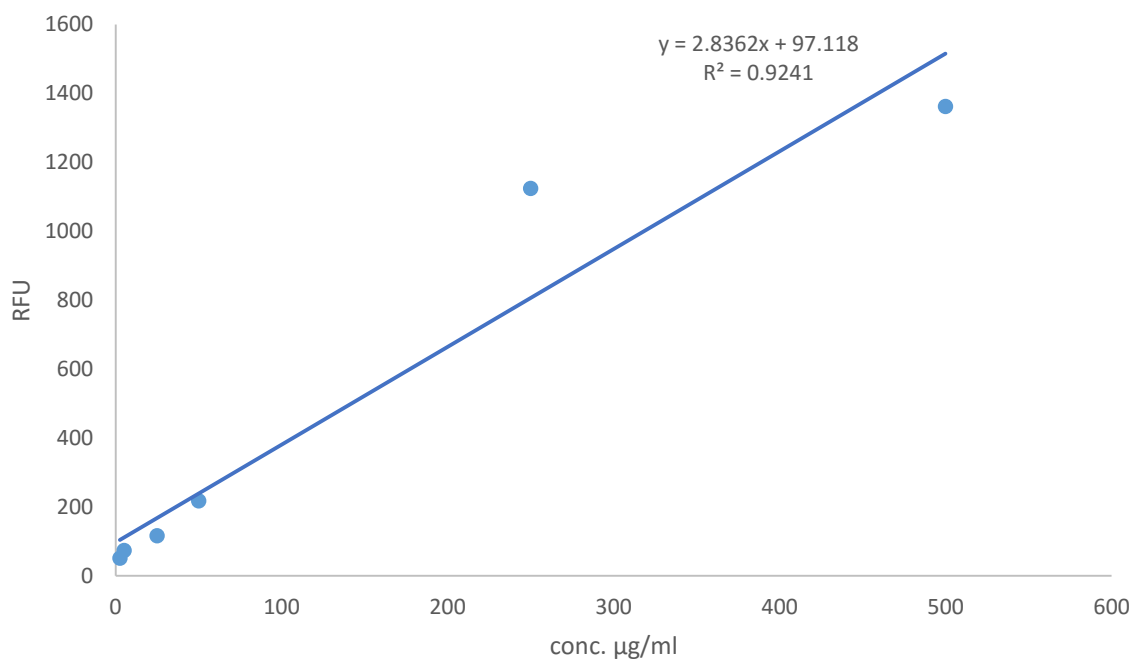


Figure 87. Plot of relative fluorescence units (RFU) versus concentration (μg/ml) for micelle **(23)**. The equation derived from the line of best fit ($y = 2.8362x + 97.118$) was used to calculate the RFU value for a 250 μg/ml solution.

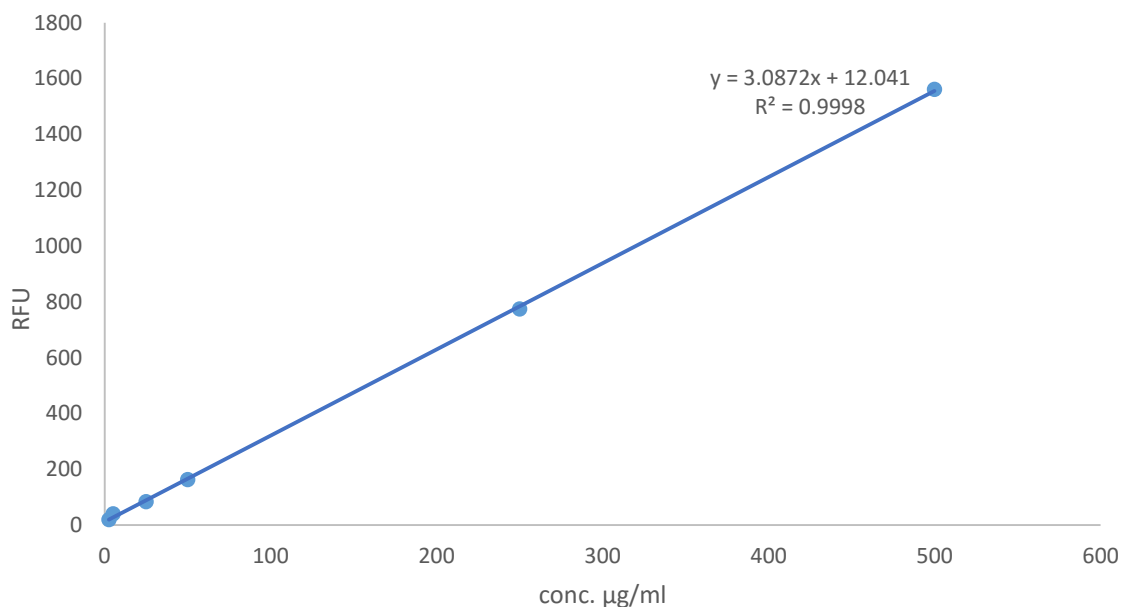


Figure 88. Plot of relative fluorescence units (RFU) versus concentration (μg/ml) for micelle **(24)**. The equation derived from the line of best fit ($y = 3.0872x + 12.041$) was used to calculate the RFU value for a 250 μg/ml solution.

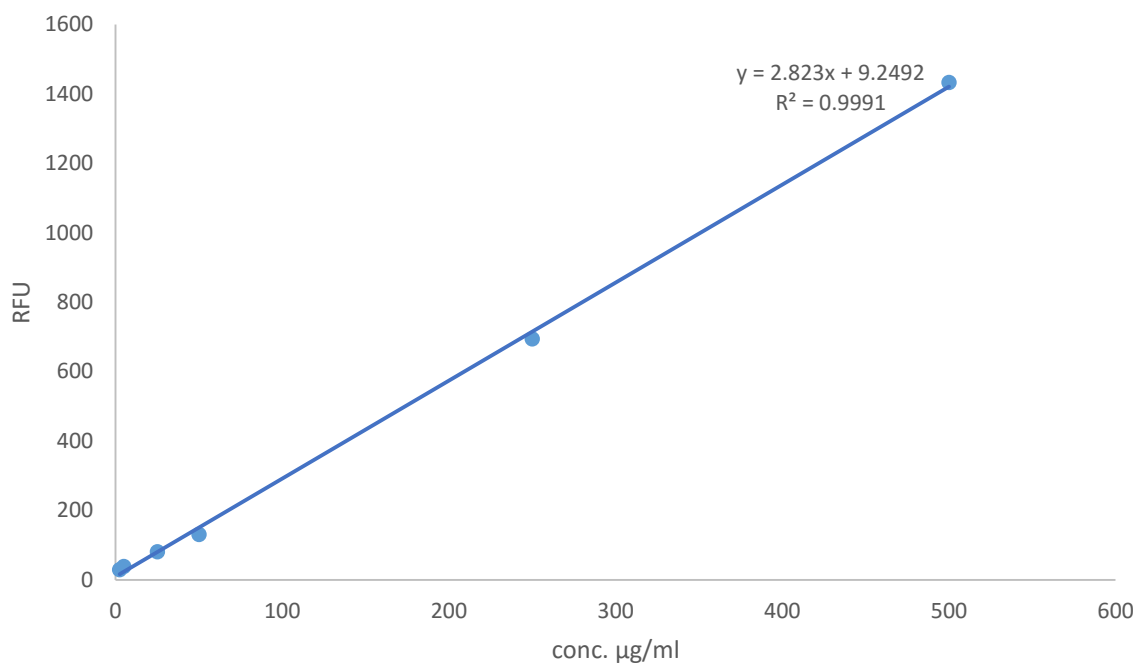


Figure 89. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle (25). The equation derived from the line of best fit ($y = 2.823x + 9.2492$) was used to calculate the RFU value for a 250 µg/ml solution.

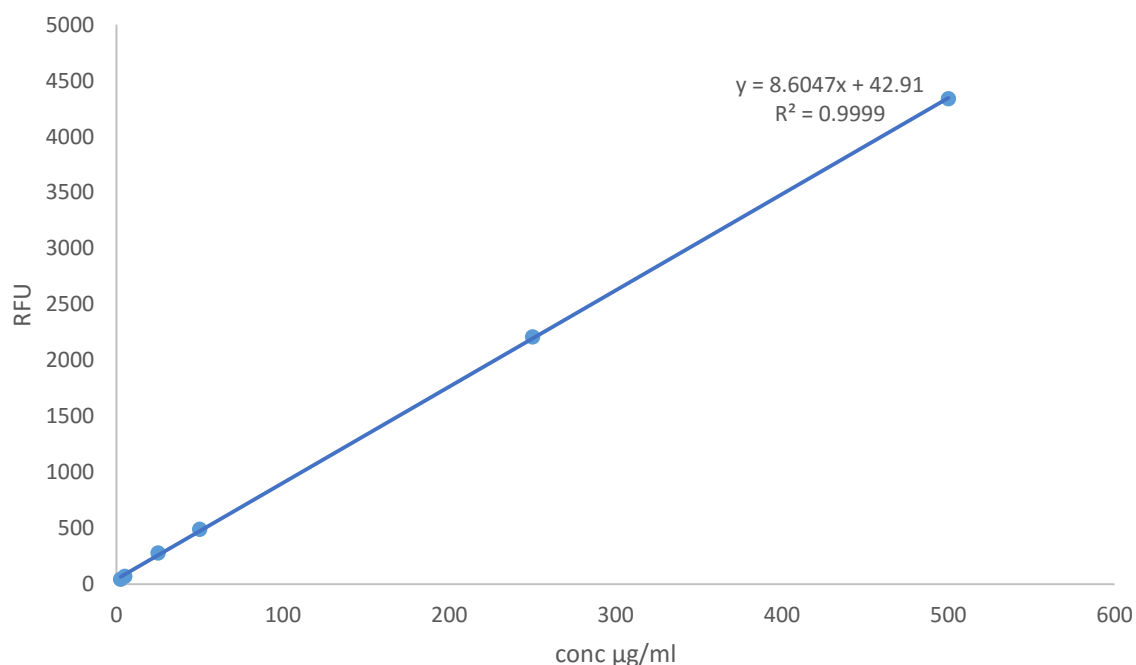


Figure 90. Plot of relative fluorescence units (RFU) versus concentration (µg/ml) for micelle (26). The equation derived from the line of best fit ($y = 8.6047x + 42.91$) was used to calculate the RFU value for a 250 µg/ml solution.

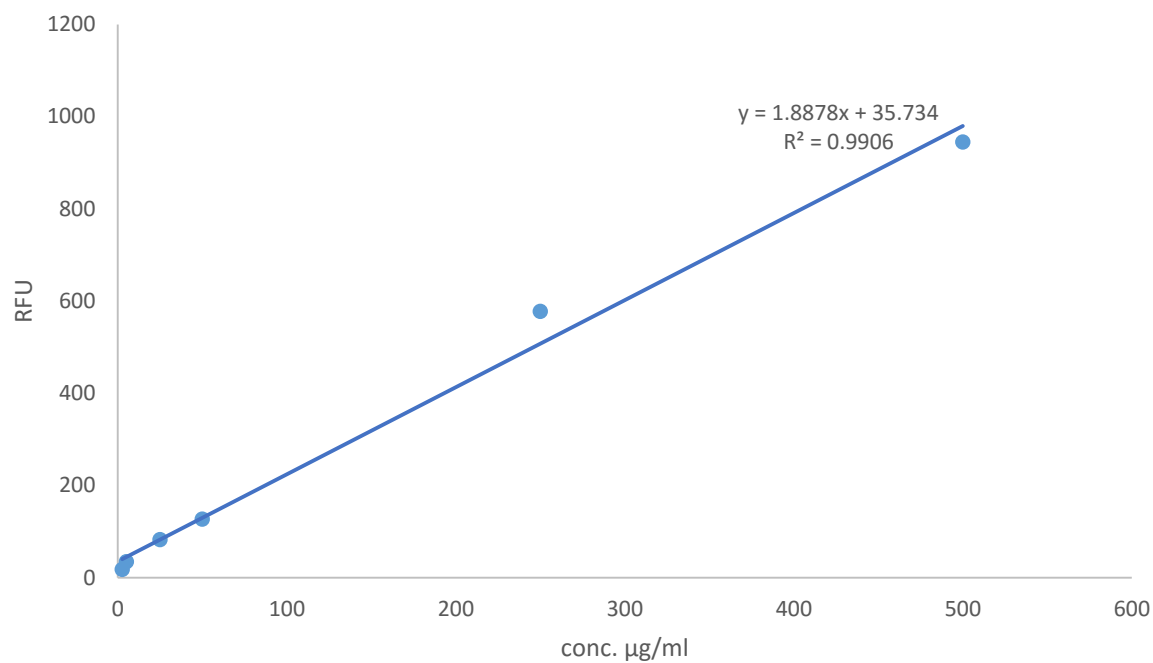


Figure 91. Plot of relative fluorescence units (RFU) versus concentration ($\mu\text{g/ml}$) for micelle **(27)**. The equation derived from the line of best fit ($y = 1.8878x + 35.734$) was used to calculate the RFU value for a 250 $\mu\text{g/ml}$ solution.